SYMBOLAE BOTANICAE UPSALIENSES XVIII:3
ARBETEN FRÅN BOTANISKA INSTITUTIONERNA I UPPSALA
UTGIVNA AV PROF. E. MELIN OCH PROF. J. A. NANNFELDT

STUDIES OF THE PHYSIOLOGY, MORPHOLOGY AND SEROLOGY OF EXOBASIDIUM

BY

KARL-RAGNAR SUNDSTRÖM

UPPSALA 1964
A.-B LUNDEQUISTSKA BOKHANDELN
(I DISTRIBUTION)

SYMBOLAE BOTANICAE UPSALIENSES

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Uppsala, February 1964. *Karl-Ragnar Sundström*

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I. GENERAL INTRODUCTION

Fuckel (1861) was the first to describe a representative of the genus *Exobasidium* but he misplaced it among the *Fungi Imperfecti* and called it *Fusidium*. A few years later Woronin (1867) published a detailed description of a fungus causing diseases in *Vaccinium vitis-idaea*. In this publication, Woronin proposed the name *Exobasidium vaccinii* and placed it in the *Basidiomycetes*. The exact taxonomic position of *Exobasidium* in that class is disputed; most systematists include it in the Homobasidiomycetidae, while others, because of its resemblance to the *Ustilaginales*, place it in the Heterobasidiomycetidae (e.g., Talbot 1954).

Since 1867 many species of the genus *Exobasidium* have been described. A characteristic feature of all of them is that they are parasitic on leaves, shoots, or flowers of woody dicotyledons. They usually attack species of the *Ericaceae*, but have also been observed with certainty on representatives of the *Theaceae*, *Lauraceae*, *Symplocaceae*, *Epacridaceae*, and *Empetraceae*. Of great interest in this connection is Gibb's investigation (1954) in which he divided up a large number of tested plants into species producing tannins and catechols or into species with no such production. The *Theaceae*, *Ericaceae*, and *Empetraceae* are among the producing species. The *Lauraceae* and *Symplocaceae* were not tested. It was suggested by Hutchinson (1948) that the *Ericaceae* has its origin in the *Theaceae*. The *Epacridaceae* is often considered the Australasian counterpart of the *Ericaceae* and many investigations support the close affinity of the two families (Paterson 1961). It can be supposed that parasites which are highly specialized adapt themselves to the physiology of their hosts. Therefore it is not surprising that groups of closely related fungi attack related plants.

In the infected parts of the plants, where the mycelium of *Exobasidium* grows intercellularly it induces cell divisions and causes an increase in cell size, resulting in galls, witches' brooms or large systemically infected shoots. Wolf and Wolf (1952) and Hirata (1957) have shown that *Exobasidium* can produce indoleacetic acid, which might at least partly explain these deformations. The enlarged parts often lose their normal mode of differentiation. The chlorophyll content is reduced and an anthocyanin coloration appears. Eftimiu and Kharbush (1927) made a cytological investigation of some *Exobasidium* species, and showed that the intercellular mycelium is septate with dicaryotic cells and with no clamps. Most species have haustoria. The width of the hyphae is 1–1.5 μ and the mycelium is concentrated in a subepidermal layer. After being intercellular for some time the mycelium penetrates or ruptures the epidermis and pushes it away. The apices of the hyphae develop into a hymenium directly on the surface of the host. In the basidia the fusion of the nuclei and the reduction division occur, and the resulting nuclei migrate through the sterigmata into the basidiospores, which are formed in varying

numbers on each basidium. The basidiospores, or the secondary spores (conidia) formed from them, initiate the disease on a new host.

The most reliable morphological characters for the delimitation of various *Exobasidium* species are the size of the basidiospores, their form, and the number of sterigmata. However, the number of sterigmata is variable. The particular host and the disease symptoms are also considered characteristic of different species, and many workers use these characters for identification of the different *Exobasidium* species. Burt (1915) and Savile (1959), among others, proposed a delimitation of species based only on the spore characters. Savile includes in *E. vaccinii* a large number of forms which are parasitic on different species of *Ericaceae*, provided that they have the following characters: "Basidiospores slenderly cylindrical to musiform. Basidiospores 8.0–15.0 (−18.0) μ long; basidia with (2–) 3–6 (–8) sterigmata." Within *Exobasidium vaccinii*, according to Savile, there are some forms causing hypertrophic spots on leaves (galls), and others attacking whole shoots (systemic attacks). The above-mentioned authors regard the disease symptoms as a growth response by the host to stimulation from the fungus. Different physiological conditions in one host or different hosts may produce different responses to attacks by the same fungus. In contrast to these authors, Juel (1912) considered it most probable that galls and systemic attacks are caused by different fungi, in spite of the fact that the morphology of the spores is almost the same. He observed that gall-producing forms could occasionally infect whole shoots and that they then caused stronger hypertrophy than other infections occurring on shoots of the same host. These others are systemic infections caused by other *Exobasidium* forms, which cannot cause the same degree of hypertrophy.

Finally, there is a general tendency to describe new species of *Exobasidium* when the fungus is found on new hosts, especially when it causes galls of peculiar appearance. As the final appearance of the attack is due to interactions between the fungus and the host, it is difficult to decide what the causes of the disease symptoms are. There remain many problems to be solved in the delimitation of species of the genus *Exobasidium*. The difficulties appear very clearly when the different proposals for the delimitation of species are studied.

The purpose of this investigation was partly to attempt to delimit the species of *Exobasidium* on a broader basis including physiological, morphological and serological characters, and partly to obtain more information about their physiology, which has been very little studied. The only previously known nutritional requirements of *Exobasidium* under controlled conditions and on a synthetic medium, were reported by Göttgens (1959) for *E. japonicum* only.

The investigation is divided into four parts:

1. Physiological studies. 2. Morphological and cytological studies. 3. Inoculation experiments. 4. Serological studies.

II. MATERIAL

Most of the *Exobasidium* strains used in the present investigation have been isolated from diseased plants, but nine strains have been obtained from Dr. Graafland, one from Dr. Montant, and one from CBS, Baarn. Isolation was according to Graafland (1953). Leaves or shoots were fixed with vaseline to the inside of the lid of a sterile petri dish, filled with a thin agar layer. Basidiospores are then discharged from the hymenium on the underside of the leaf onto the agar, where they germinate and grow into colonies. By turning the lid round at short intervals during discharge of the spores, a greater dispersal is obtained. After microscopic examination through the bottom of the petri dish, one single basidiospore, if possible, or one solitary colony was isolated from the dish. These isolates were transferred to nutrient solutions where they grew and produced conidia. Suspensions of these conidia were plated out with an agar medium in petri dishes. A solitary colony was then isolated again. In doubtful cases the procedure was repated once more in order to be absolutely sure of getting monospore cultures. It is generally easy to obtain pure cultures of *Exobasidium*. As a rule 2–10 hours will suffice for a large quantity of spores to be discharged from the gall, and usually no spores from contaminating fungi are disseminated before the galls are removed from the lid. Low temperatures are favourable to spore discharge. For many strains 15–20°C is the best temperature and at 25°C there is already a clear decrease in the release of spores. The spores and conidia of these forms do not germinate or grow at 25°C. Difficulties arise when plants with old *Exobasidium* galls are collected. These are often overgrown by contaminating fungi. There are also more contaminations when immature galls are collected, because it takes a longer time before the ejection of the spores begins. In order to assess the variation within the species of *Exobasidium*, strains obtained from different geographic areas were studied, e.g., strains from the south of Germany and from the mountains of Northern Sweden.

A list of the species studied, year and place of isolation, their hosts, and the disease symptoms, follows below (Table 1). The nomenclature is as far as possible in accordance with Fungi Exsiccati Suecici, Praesertim Upsalienses.

The stock-cultures were maintained on agar slants (20 g malt, 12 g agar, 0.4 g N–Z case and 0.2 g yeast extract per 1), which were stored in a deep freeze at −20°C.

Table 1. A list of the tested *Exobasidium* strains.

Exobasidium species		Host	Year and place of isolation	Disease symptoms
E. angustisporum Linder	1	*Arctostaphylos alpina*	A 1959	S
	2		A 1962	S
	3		A 1962	S
E. cassiopes Peck	1	*Cassiope tetragona*	A 1959	S
	2		A 1962	S
E. japonicum Shir.	1	*Azalea*	1961	G
	2	*Azalea* cultivar 'Esmeralda'	Gr.	
	3	*Azalea* cultivar 'Hexe'	Gr.	
	4	*Azalea*	CBS	
E. Karstenii Sacc. & Trott.	1	*Andromeda polifolia*	A 1959	S
	2		A 1959	S
	3		Ö 1959	S
	4		U 1959	S
	5		Sw 1961	S
	6		St 1961	S
E. myrtilli Siegm.	1		U 1959	S
	2		St 1961	S
E. myrtilli Siegm.	1	*Vaccinium myrtillus*	A 1959	S
	2		A 1959	S
	3		A 1959	S
	4		Ö 1959	S
	5		St 1961	S
E. myrtilli Siegm.	1	*Vaccinium vitis-idaea*	A 1959	S
	2		A 1959	S
	3		Ö 1959	S
	4		U 1960	S
	5		St 1961	S
	6		U 1962	DI
	7		U 1962	DI
E. myrtilli Siegm.	1	*Vaccinium uliginosum*	A 1962	S
E. rhododendri Cram.	1	*Rhododendron ferrugineum*	M	
E. vaccinii (Fuck.) Woron.	1	*Vaccinium vitis-idaea*	U 1958	G
	2		U 1959	G
	3		V 1959	SG
	4		V 1959	G
	5		U 1960	G
	6		St 1961	G
	7		St 1961	G
	8		Sw 1961	G
	9		Sw 1961	G
	10		Ö 1961	G
	11		Ka 1961	G
	12		H 1961	G

Table 1 (*cont.*)

Exobasidium species			Host	Year and place of isolation	Disease symptoms
E. vaccinii (Fuck.) Woron.		13	*Vaccinium vitis-idaea*	K 1961	G
		14		U 1961	G
		15		Gr.	
		16		Gr.	
		17		U 1962	DI
		18		U 1962	DI
E. vaccinii (Fuck.) Woron.		1	*Arctostaphylos uva-ursi*	U 1959	g
		2		U 1959	g
		3		U 1961	g
		4		U 1961	S
		5		U 1962	g
E. vaccinii (Fuck.) Woron.		1	*Vaccinium myrtillus*	U 1960	g
E. vaccinii (Fuck.) Woron.		1	*Vaccinium oxycoccos*	Sw 1961	g
		2		U 1961	g
		3		U 1962	g
		4		U 1962	g
E. vaccinii (Fuck.) Woron.		1	*Vaccinium uliginosum*	U 1960	g
		2		St 1961	g
		3		Sw 1961	g
		4		U 1961	g
		5		U 1961	g
		6		U 1962	g
E. vexans Mass.	(B_1)	1	*Camellia sinensis*	Gr.	
	(B_{12})	2		Gr.	
	(B_{27})	3		Gr.	
	(B_{28})	4		Gr.	
	(B_{76})	5		Gr.	
E. uvae ursi (Maire) Juel		1	*Arctostaphylos uva-ursi*	U 1959	W
		2		U 1959	W

The fungi were collected in the vicinity of the following places (Fig. 1):

A = Abisko	Ky = Kyrkhult	U = Uppsala
H = Hok	St = Starnberg	V = Vansbro
K = Katrineholm	Sw = Schwarzwald	Ö = Örnsköldsvik

CBS Pure culture received in 1962 from Centraalbureau voor Schimmelcultures, Baarn.
Gr Pure culture received in 1962 from Dr. Graafland, Baarn.
M Pure culture received in 1962 from Dr. Montant.
S Systemic attacks on whole shoots.
G A localized leaf spot with a hypertrophic gall.
SG Shoot galls.
DI Double infection. A leaf gall on a systemically infected shoot without hypertrophy.
g A small localized leaf spot with a small hypertrophic gall.
W Witches' brooms.

Fig. 1. Map of localities from which *Exobasidium* strains have been isolated.

Glossary of some of the words used in the account of the present investigation:

Conidial form: The basidiospores of some *Exobasidium* strains germinate and produce conidia which continue to grow by budding like yeast cells.

Host-race: *Exobasidium* strains isolated from the same host species causing similar disease symptoms belong to the same host-race. *E. vaccinii* "vit.id.", *E. vaccinii* "Arct." and *E. myrtilli* "vit.id." are examples of three different host-races, isolated from *Vaccinium vitis-idaea*, *Arctostaphylos uva-ursi*, and *Vaccinium vitis-idaea*, respectively.

Mycelial form: The basidiospores of some *Exobasidium* strains germinate with long germ-tubes. These strains grow in a mycelial manner on solidified media and if transferred into nutrient solution a mixture of hyphae and conidia is produced.

Species: When this word is used with reference to *Exobasidium* strains the nomenclature listed in Table 1 is applicable.

Strain: The descendants of a single isolation in pure culture.

III. METHODS

A. Methods of cultivation

The following nutrient solutions modified from L. Fries (1955) have been used:

Medium 1

Solution A		Solution B	
D-Glucose	20 g	$CaCl_2$	0.10 g
L-Asparagine · H_2O	1.4 g	$MgCl_2 \cdot 6H_2O$	0.41 g
KH_2PO_4	0.35 g	Ferricitrate	5.31 mg
K_2HPO_4	0.15 g	Citric acid	5.31 mg
$Na_2SO_4 \cdot 10\ H_2O$	0.5 g	$MnSO_4 \cdot 4H_2O$	4.43 mg
Thiamine	0.1 mg	$ZnSO_4 \cdot 7H_2O$	4.05 mg
Dist. water	1000 ml	Dist. water	50 ml

Medium 2

Solution A		
D-Glucose	20 g	
L-Asparagine · H_2O	1 g	
$(NH_4)_2$-tartrate	0.5 g	
Citric acid · H_2O	6.17 g	+ Solution B
$Na_2HPO_4 \cdot 2H_2O$	7.5 g	
KH_2PO_4	0.35 g	
$Na_2SO_4 \cdot 10H_2O$	0.5 g	
Thiamine	0.1 mg	
Dist. water	1000 ml	
pH = 4.2		

Medium 3

Solution A		
D-Glucose	20 g	
NH_4NO_3	0.6 g	
$NaH_2PO_4 \cdot H_2O$	4.1 g	
K_2HPO_4	0.15 g	+ Solution B
$Na_2SO_4 \cdot 10H_2O$	0.5 g	
Thiamine	0.1 mg	
Dist. water	1000 ml	

pH of solution A was adjusted to 4.2 with HCl.

Medium 4

Glucose	10.00 g
Casamino acids	5.00 g
K_2HPO_4	0.75 g
NaCl	1.00 g
$MgSO_4$	1.50 g
Ferricitrate	0.02 g
Thiamine	0.1 mg
Vitamin mixture	1 ml
Dist. water	1000 ml

pH of medium 4 was adjusted to pH 6.0.

Veronal buffer.

Diethylbarbituric acid	0.575 g
Sodium diethylbarbiturate	0.375 g
NaCl	8.5 g
Dist. water	1000 ml

Vitamin stock solution in mg per ml

Riboflavin	0.20	Biotin	0.01
Pyridoxine	0.15	Inositol	0.40
Niacin	0.15	Choline	1.00
Ca-pantothenate	0.15	Folic acid	0.02
p-aminobenzoic acid	0.20		

When used, one ml of the vitamin stock solution was added to 1 l of nutrient solution.

The concentration of agar in agar medium was 1.2 %. The chemicals used were of the highest purity commercially available.

In the experiments all isolates with conidial growth were cultivated in test-tubes 20 mm wide, and those with mycelial growth in 100 ml Jena Erlenmeyer flasks. For each test at least 3 test-tubes or 4 flasks were used. The test-tubes contained 10.5 ml and the flasks 21 ml of nutrient solution, of which 0.5 ml and 1 ml, respectively, was the quantity of inoculum. During sterilization of the media, the solutions A and B were autoclaved separately for 5–10 minutes at 110–120°C. The solutions of vitamins and other thermolabile compounds were sterilized by filtration through glass filters (Jena G 5). Inocula of the forms with conidial growth were prepared by taking 0.25 ml of conidial suspension from a growing culture with a Z-value lying between 1000 and 1500 (see below). This suspension was added to 50 ml of solution B. One ml, and 0.5 ml, of this diluted spore suspension was added to solution A at inoculation of the flasks and tubes, respectively. As the forms with mycelial growth produced very few conidia, a suspension of mycelia and conidia was centrifuged, washed once, and then added to solution B for use as inocula in the same way.

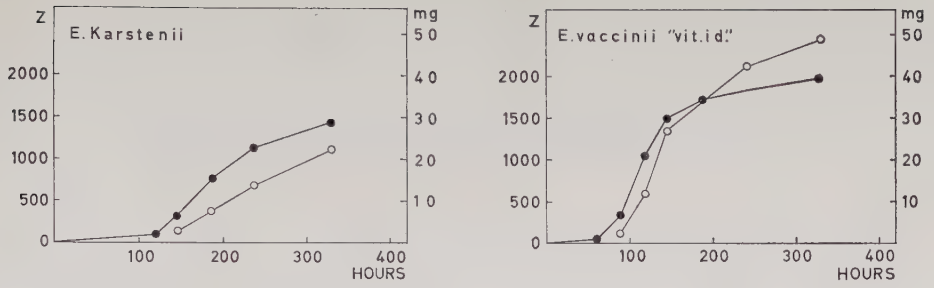

Fig. 2. The growth of *E. Karstenii* 1 and *E. vaccinii* "vit. id." 1 plotted as turbidimetric values —●— and as dry weight (mg/10 ml) —○—.

After mixing all the constituents, the initial pH of the solution was determined by a Radiometer pH-meter 22 using a glass electrode. Each tube was furnished with 2 small glass beads which prevented the conidia from aggregating. The culture tubes were continuously shaken at 19°C and growth was determined turbidimetrically using an Engel colorimeter (Kipp, Delft) at 610 mμ. As a measure of the growth the Z-value was used.

$$Z = \text{extinction value} \times 1000$$

When an experiment was finished each culture was examined microscopically and those with contaminations were discarded.

The mycelium of the mycelial forms was cultured in flasks at 19°C without shaking and was harvested by collection onto filter paper. It was washed, dried at 100°C for about 24 hours, and weighed.

B. Calibration of the turbidimetric values

The turbidimetric values for growth of one species growing in a nutrient solution as separate cells and of another showing a tendency to aggregate (*E. vaccinii* "vit.id." and *E. Karstenii*) were correlated with dry weight. Medium 2 was used.

Mean values of the turbidimetric measurements and the corresponding dry weights can be seen in Fig. 2. There was a good correlation between the two methods of growth determination, but at the highest densities (above $Z = 1000$) the turbidimetric values were too low.

In the following experiments it was not necessary for the measurements of growth to give precise quantitative information. In most experiments, controls were used that gave optimal or no growth. By comparing the growth of the controls with growth on the different test media, information was obtained about the ability of the various forms to utilize various compounds and about the stimulating effects of the compounds.

In a few cases there was pigment production that made the turbidimetric values unreliable. This is mentioned in the text where it is relevant.

IV. PHYSIOLOGICAL STUDIES

A. Introduction

The physiological study of *Exobasidium* can be divided into two sections. The first section includes an evaluation of the best conditions for cultivation. Different synthetic nutrient solutions were tested and optimum temperatures and pH were determined. The second section is concerned with the search for physiological characters useful as discriminants in taxonomic separation of the taxa. In these experiments the ability of the different strains to utilize different carbon and nitrogen sources was tested, as well as their ability to synthesize vitamins. In the same way as morphological characters, physiological characters may differ among different forms of the same species. By starting with several strains from each species and with several species represented, it ought to be possible to determine which physiological characters are stable and useful for characterization or identification of a taxonomic group. For organisms only poorly differentiated morphologically from one another, physiological characters have previously been a great help in identification, e.g., in bacteria, yeasts and *Fungi Imperfecti*.

B. Growth at different pH-values

The effect of pH on growth in synthetic medium was studied using different buffers. In these experiments the components of the buffer system were dissolved in 50 % of the amount of water in solution A. After mixing the buffer components to the desired pH, the buffers were autoclaved in the tubes. The solution A of medium 1 with half the quantity of water and solution B were autoclaved and afterwards these solutions were added to the buffers in the tubes and the initial pH was determined. The stock-buffers used (mixed with the same quantity of nutrient solution), the concentration of their components, and the pH values obtained were as follows:

Citrate-phosphate buffer. 0.1 M solution of citric acid 0.2 M solution of $Na_2HPO_4 \cdot 2H_2O$	pH 2.8–7.0
Citrate buffer. 0.1 M solution of citric acid 0.1 M solution of sodium citrate	pH 3.8–5.8
Phosphate buffer. 0.1 M solution of $NaH_2PO_4 \cdot H_2O$ 0.1 M solution of $Na_2HPO_4 \cdot 2H_2O$	pH 7.0–7.7

Phthlate-hydrochloric acid buffer pH 2.2–3.2
0.2 M solution of potassium acid phthlate
0.2 M HCl

Tris (hydroxymethyl) aminomethane buffer pH 7.3–8.4
0.04 M solution of tris (hydroxymethyl) aminomethane
0.04 M HCl

The composition of the buffers was derived from Colowick and Kaplan (1955).

E. vaccinii "vit.id." was cultivated on the differently buffered media. Within the investigated pH-ranges only the first three of the abovementioned buffers permitted growth while the others were toxic or gave a pH that inhibited growth. To find out the effect of the different concentrations of hydrogen ions on *Exobasidium*, the following experiment was carried out. Citrate-phosphate buffers were used in the media between pH 2.8–7.0 and phosphate buffers at pH 7.0–7.7. Both the stock-buffers were diluted with nutrient solution to half the ion strength. Fig. 3 shows the growth of three *Exobasidium* strains at different pH values. The best conditions for growth of *E. vaccinii* "vit.id." and *E. angustisporum* were

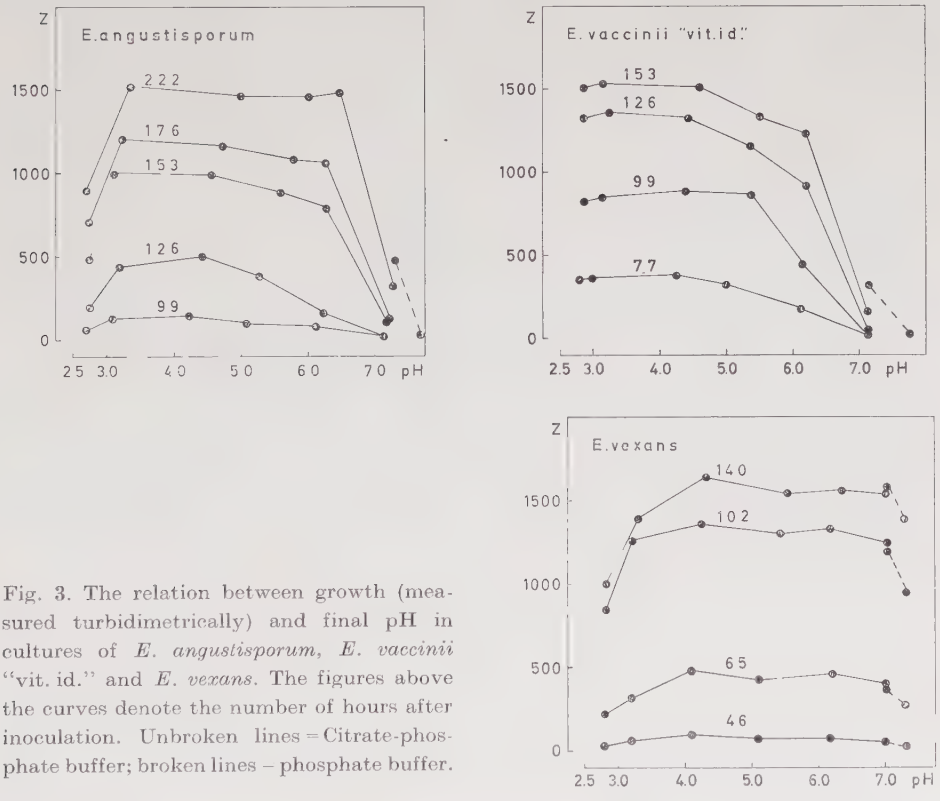

Fig. 3. The relation between growth (measured turbidimetrically) and final pH in cultures of *E. angustisporum*, *E. vaccinii* "vit. id." and *E. vexans*. The figures above the curves denote the number of hours after inoculation. Unbroken lines = Citrate-phosphate buffer; broken lines – phosphate buffer.

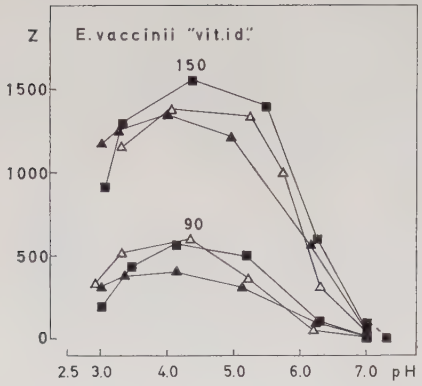

Fig. 4. The relation between growth (measured turbidimetrically) and final pH in cultures of *E. vaccinii* "vit. id." with different sources of nitrogen. The figures above the curves denote the number of hours after inoculation. —■— = asparagine; —▲— = $(NH_4)_2$-tartrate; —△— = KNO_3.

obtained within a pH-range of 3.0–6.0. Outside this range, growth decreased very rapidly. Most of the tested strains had a pH optimum between 3.0–6.0, e.g., *E. Karstenii*, *E. uvae ursi*, *E. vaccinii* "Arct.", and and *E. vaccinii* "ulig.". A wider pH optimum range for growth, 3.0–7.0, was found in *E. vexans* (Fig. 3) and *E. japonicum* (Fig. 5). *E. cassiopes* and *E. myrtilli* occupied an intermediate position.

Among the pH-dependent factors that can affect growth, the following have to be considered: enzyme action, uptake of nutrients, metal solubilities and toxicity. The uptake of asparagine or the enzymatic capacity to utilize it may determine the dependency of growth on pH. In a weakly alkaline medium with asparagine, *Aspergillus niger* utilized amide nitrogen rapidly but amino nitrogen very slowly (Schmalfuss and Mothes 1930). In acid medium, amino as well as amide nitrogen was a good nitrogen source. These authors suggested that it was possible that the asparaginase active under acid conditions was not identical with the asparaginase active in the alkaline medium. The latter enzyme was intracellular and its activity began at pH 6.5 and increased very rapidly at higher pH.

The absence of growth of *Exobasidium* above pH 7.0 may be caused by lack of an asparaginase. To eliminate this possibility and the possibility that asparagine was not readily taken up, this nitrogen source was exchanged for ammonium tartrate or KNO_3. However, this substitution caused no appreciable change in the pH dependency of the growth of *E. vaccinii* "vit.id." (Fig. 4).

Further it is not very likely that pH-conditioned differences in the uptake of thiamine by the organisms or the destruction of the vitamin during the heat sterilization limited growth, because the thiamine heterotrophic species, *E. vexans*, had a greater pH tolerance than the vitamin-autotrophic species, e.g., *E. uvae ursi*.

No media with pH values exceeding 7.7 were tested because above this pH a precipitate was formed.

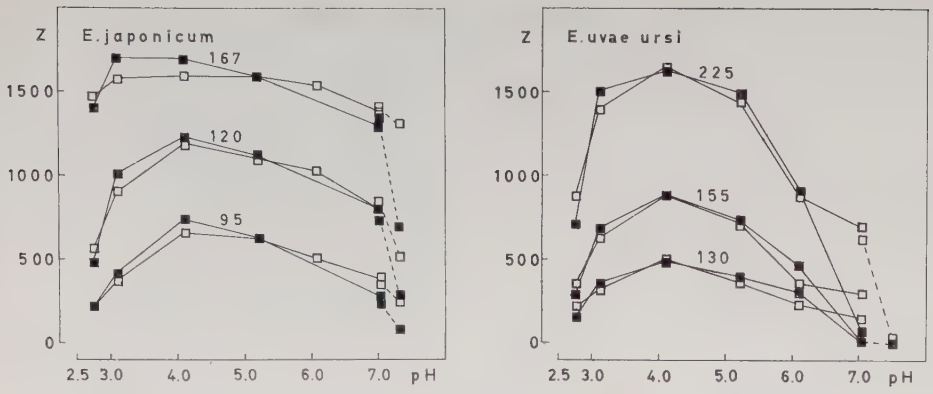

Fig. 5. The relation between growth (measured turbidimetrically) of *E. japonicum* and *E. uvae ursi* and initial pH of the media. The figures above the curves denote the number of hours after inoculation. —■— = stock buffer diluted to 50 %; —□— = stock buffer diluted to 25 %.

Some microorganisms are sensitive to high concentrations of nutrient ions in the media. Crasemann (1954) showed that *Chytridium* and *Macrochytrium* could not grow when the concentration of phosphate in the medium exceeded 0.005 M.

As both the citrate-phosphate and phosphate buffers caused an increased concentration of phosphate at the higher pH there was reason to test lower concentrations of the buffers. In these experiments the ionic strength of the stock-buffers was reduced to 50 or 25 per cent. The most diluted buffer had a very low buffer capacity. As can be seen from Fig. 5, the most concentrated buffer caused a growth reduction at the highest pH. This sensitivity to high concentrations of phosphate at high pH occurred in all the tested strains. At pH 4.2, the pH which has been chosen for most of the experiments, the more concentrated citrate-phosphate buffers caused no inhibition. The buffer used in medium 2, pH 4.2 (p. 13), corresponds to the 50 per cent diluted stock-buffer. This buffer was not perfect because it had a rather low buffer capacity at pH 4.2. In extreme cases the pH changed to about pH 3.0 or 6.0 at the end of the experiment. The nitrogen source had the greatest influence on this pH shift, NH_4^+ causing the lowest pH and NO_3^- the highest final pH. However, these pH shifts did not appear to be inhibitory to growth, because they occurred within the pH range where the growth was good.

C. Growth at different temperatures

Information about the temperature tolerances of *Exobasidium* is meagre. Graafland (1953) found room temperature, 17–20°C, optimal for the growth of *E. vaccinii* parasitic on *Vaccinium vitis-idaea*. A constant temperature of 28°C was unfavourable. For *E. japonicum* 20–22°C was optimal (Graafland 1960). Accord-

Table 2. The optimum and maximum temperatures for growth of different *Exobasidium* strains.

Strains	Optimum temperature °C	Maximum temperature °C	Place of isolation
E. vexans B_1		34	
E. japonicum 1		29	
E. vaccinii			
— "Arct." 2		29	Uppsala
— "Arct." 5		29	Uppsala
— "myrt." 1	19–25	29	Uppsala
— "vit. id." 7	19–25	29	Starnberg
— "vit. id." 10	19–25	29	Örnsköldsvik
— "vit. id." 11		29	Katrineholm
E. Karstenii 2	19–25	27	Abisko
E. Karstenii 3	19–25	27	Örnsköldsvik
E. Karstenii 5	19–25	27	Schwarzwald
E. myrtilli			
— "Andr." 1	19–25	27	Uppsala
— "Andr." 2		27	Starnberg
E. vaccinii			
— "oxyc." 1		27	Schwarzwald
— "oxyc." 4		27	Uppsala
— "ulig." 1		27	Uppsala
E. uvae ursi 1		27	Uppsala
E. uvae ursi 2		27	Uppsala
E. angustisporum 1	14–19	25	Abisko
E. angustisporum 3		25	Abisko
E. cassiopes 1	14–19	25	Abisko
E. myrtilli			
— "vit. id." 3	14–19	25	Örnsköldsvik
— "vit. id." 4	19	25	Uppsala
— "vit. id." 5		25	Starnberg
— "vit. id." 6		25	Uppsala
— "ulig." 1		25	Abisko
E. rhododendri 1		25	
E. myrtilli			
— "myrt." 2	14–19	23	Abisko
— "myrt." 3		23	Abisko
— "myrt." 4	14–19	23	Örnsköldsvik
— "myrt." 5		23	Starnberg

ing to Lockhart (1958) the optimal temperature for growth of the *Exobasidium* species parasitic on *Vaccinium myrtilloides* Michx. is 20–24°C and the maximum temperature 32.8°C.

During isolation of the *Exobasidium* strains in the present investigation it was

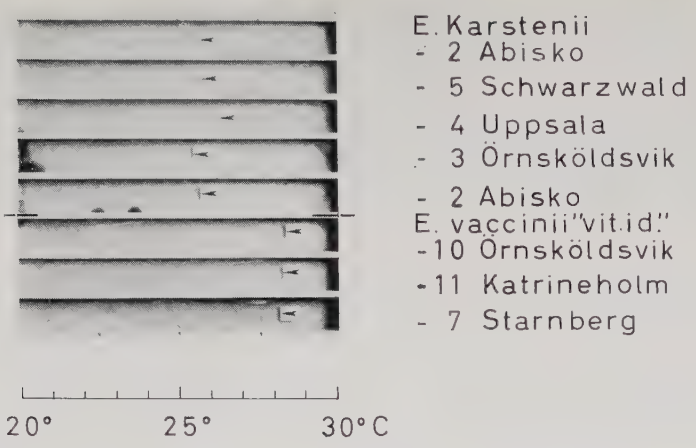

Photo 1. Growth of four different strains of *E. Karstenii* and three different strains of *E. vaccinii* "vit. id." on a temperature-gradient plate. The arrows indicate the borderline where growth stopped.

observed that for some strains a temperature of 25°C was too high for good growth, while the growth of other strains was not inhibited at that temperature. Therefore a temperature test was made to determine the temperature tolerances of *Exobasidium*. To find out the temperature optima for growth of the different strains, the fungi were cultivated on medium 2 in shake cultures at 5, 14, 19, 25, and 29°C. The maximum temperature for each strain was found by studying the growth on malt agar media in petri dishes at 20, 23, 25, 27, 29, 32, and 34°C. It can be seen in Table 2 that the host-races responded differently to the temperatures and that the growth usually ceased at temperatures slightly above the optimum. Although the values of optimum and maximum temperatures for growth in Table 2 were obtained under different conditions, the maximum temperatures found with the two methods agreed. In several cases, the host-races were represented by different geographical isolates, but even then all the strains of each host-race had the same maximum temperature. However, as there were two or three degrees between the tested temperatures, small variations in tolerance among the geographical strains might not have been observed.

Experiments with a temperature-gradient agar plate (Fries 1963) gave more precise information about the variations in temperature tolerance within two species. Across the plate there was a continuous gradient of temperature from one end of the plate to the other. In my tests the plates were regulated to temperatures between 20 and 30°C. Molten malt agar containing the conidial suspension was poured into the sterile, closed, temperature-gradient plate. After 2 weeks' incubation, the plate was opened and the maximum temperature for growth could be inferred from the borderline where growth ceased. In spite of the large climatic

differences between the sites of origin of the host-races—one at 500 m above sea level, north of the arctic circle, and the other at sea level in the south of Sweden —the results of this test indicated that temperature tolerance within the host-races was characteristic, with less than one degree variation in the maximum temperature for growth (Photo 1).

Hence, it seems that the maximum temperatures for growth may be of taxonomic value.

Unless stated otherwise, the tests that are reported in the following pages have been carried out at 19°C.

D. The utilization of nitrogen

Only little information on the nitrogen metabolism of *Exobasidium* is available in the literature. Wolf and Wolf (1952) and Hirata (1957) reported that they cultivated *Exobasidium* on Czapek's medium with KNO_3 as the source of nitrogen. In Hirata's investigation, which included a large number of *Exobasidium* species, it was observed that the growth rate was higher when nitrate was exchanged for L-tryptophan. According to Göttgens (1959), organic nitrogen was also superior to inorganic nitrogen for *E. azaleae* (*E. japonicum*). For this species DL-glutamic acid seemed to support better growth than L-asparagine, glycine and DL-alanine.

Some investigators have used differences in the utilization of nitrogen for the delimitation of taxa. Stelling-Dekker (1931) distinguished the two yeast genera *Pichia* and *Hansenula* by their different capacities to assimilate nitrate. Wickerham's studies of *Hansenula* (1951) further confirmed the value of this character; all of the 15 investigated species being capable of utilizing nitrate. Mix (1953) studied the ability of 79 isolates, comprising 27 species and 57 host forms, of the ascomycete genus *Taphrina* to utilize eight different ammonium compounds, four nitrates, urea, peptone, asparagine and 17 amino acids. The capacity for utilization of different sources of nitrogen was characteristic of the different species. Within a species some host forms agreed closely in nitrogen utilization, while others showed various degrees of difference. Sometimes these physiological differences within the species were accompanied by morphological differences, which in some cases might justify a division of the species.

Results of such promising value for systematic classification are far from gene-

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Fig. 6. The utilization of some nitrogen and carbon compounds by *Exobasidium* strains. The growth curves are based on measurements of turbidity and the columns on dry weights (mg/flask). A bar in the left of the squares indicates harvest after 15–20 days; in the right after 55–65 days. In the nitrogen tests glucose was used as the source of carbon, in the carbohydrate tests NH_4NO_3 was the source of nitrogen. Medium 2 and 3, respectively, were used. The measurements of turbidity were made at least twice a week; the curves are of growth-values up to Z = 1600.

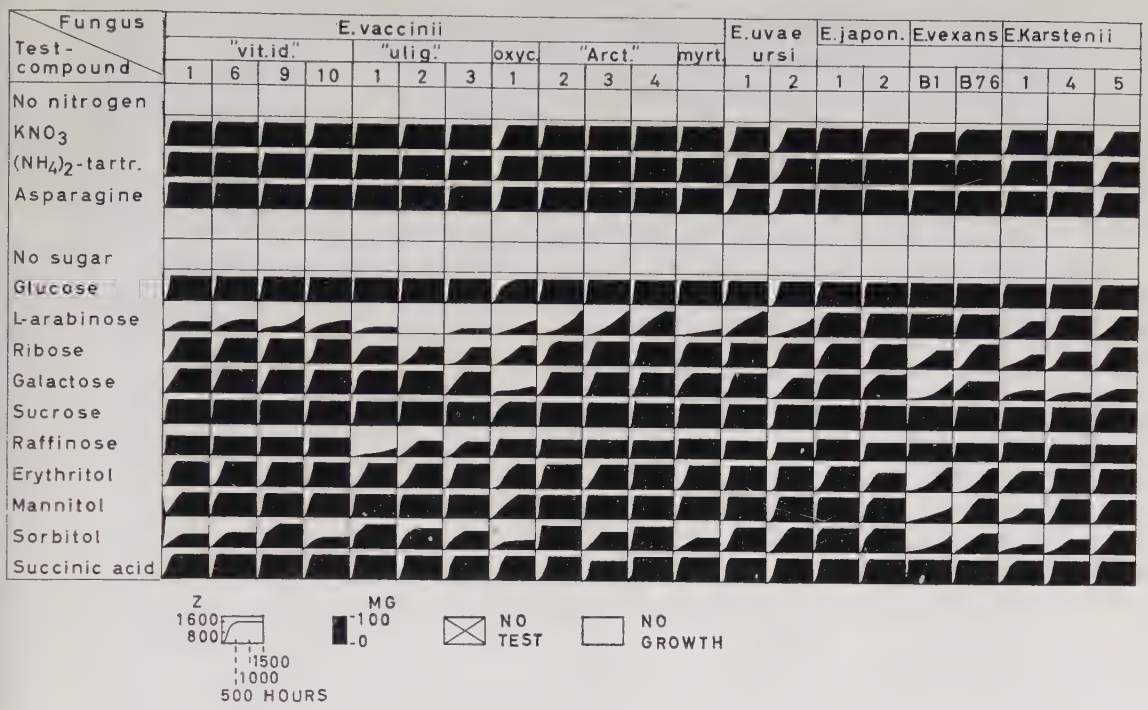

Fungus
Test-compound
E.vaccinii
"vit.id."
"ulig."
oxyc.
"Arct."
myrt.
E.uvae ursi
E.japon.
E.vexans
E.Karstenii
1 6 9 10 1 2 3 1 2 3 4 1 2 1 2 B1 B76 1 4 5
No nitrogen
KNO3
(NH4)2-tartr.
Asparagine
No sugar
Glucose
L-arabinose
Ribose
Galactose
Sucrose
Raffinose
Erythritol
Mannitol
Sorbitol
Succinic acid
Z
1600
800
1500
1000
500 HOURS
MG
100
0
NO TEST
NO GROWTH

Fig. 6.

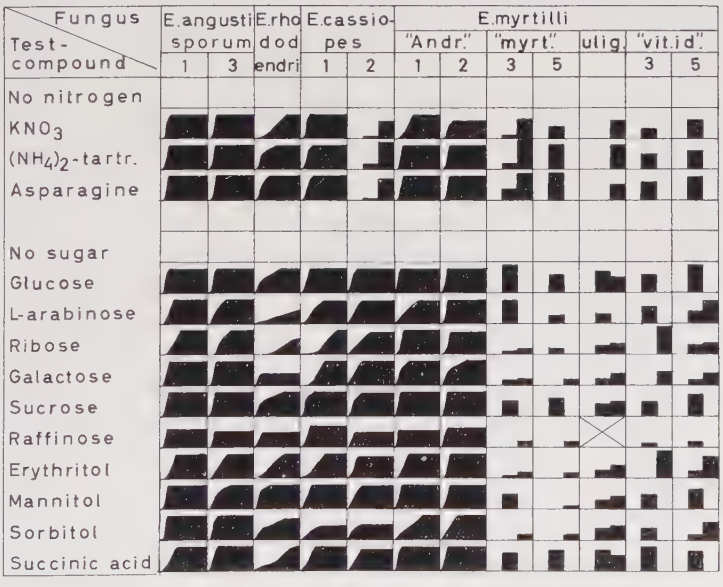

Fungus
Test-compound
E.angusti sporum
E.rhododendri
E.cassiopes
E.myrtilli
"Andr."
"myrt."
ulig.
"vit.id".
1 3 1 2 1 2 3 5 3 5
No nitrogen
KNO3
(NH4)2-tartr.
Asparagine
No sugar
Glucose
L-arabinose
Ribose
Galactose
Sucrose
Raffinose
Erythritol
Mannitol
Sorbitol
Succinic acid

Fig. 6.

ral. in fungi, as mutations have often changed the utilization of both organic and inorganic nitrogen (Cochrane 1958, p. 243).

Bolard (1956, 1957) has carried out a comprehensive chromatographic analysis of xylem sap from a large number of plants. He found that glutamine and asparagine were the major nitrogen constituents in the sap. Hence, asparagine was chosen as the organic nitrogen source in tests of the ability of *Exobasidium* strains to utilize organic and inorganic nitrogen. In this experiment medium 2 was used, from which the nitrogen sources were excluded. Instead of the $(NH_4)_2$-tartrate, K-Na-tartrate was added. All *Exobasidium* isolates were tested on asparagine, $(NH_4)_2$-tartrate, and KNO_3. The different nitrogen compounds added to the modified medium 2 contained a total of 0.29 g of nitrogen per l. The solutions were autoclaved. Prior to the physiological test, the inoculation materials were cultivated on asparagine-ammonium medium. From Fig. 6, where only some of the strains are represented, it can be seen that inorganic nitrate nitrogen and ammonium nitrogen, as well as organic nitrogen in the form of asparagine, were all good nitrogen sources except for *E. rhododendri* where the assimilation of nitrate was slow.

Under the conditions of the test, asparagine and ammonium-tartrate supported growth better than KNO_3, with the exception of *E. cassiopes* 1, for which KNO_3 was superior.

The higher basidiomycetes often lack the capacity to utilize nitrate (Cochrane 1958). The result of this nitrogen test, complemented by Hirata's studies, indicates that utilization of nitrate is general in *Exobasidium*. In the previously mentioned study by Göttgens (1959), the poor growth on media with an inorganic nitrogen source may have been due to the cultivation of the fungi on an incomplete medium.

In controls with asparagine sterilized by filtering, growth was as good as that with autoclaved asparagine.

The media that have been used contained a citric acid buffer system besides organic nitrogen. When, in the following experiments, the ability of the *Exobasidium* strains to utilize different carbohydrates was to be tested, it did not seem advisable to use nutrient media with organic compounds other than the carbon sources to be tested. However, preliminary experiments of ten day's duration showed that *E. vaccinii* "vit.id." could not utilize either tartrate or citric acid and that asparagine (1 g/l) supported only a poor growth. On the other hand, it is known that more or less utilizable carbon sources can stimulate the assimilation of an unutilizable sugar (Lindberg 1963). Steinberg (1939) observed that two carbon sources that were not individually utilized gave good growth when they were mixed. In other cases the presence of one carbohydrate can inhibit the utilization of another. These results show that it is necessary to exclude all organic compounds from the media, except the vitamins.

Table 3. The pH at harvest in cultures containing different nitrogen sources.

The pH at inoculation was 4.1. See also Fig. 7.

Strains	Final pH					
	NH_4Cl	$(NH_4)_2SO_4$	$NH_4H_2PO_4$	NH_4NO_3	$(NH_4)_2$tart.	KNO_3
E. vaccinii, "vit. id." 1	2.5	2.5	3.5	4.9	2.9	6.4
E. Karstenii 1	2.5	2.5	3.4	5.1	2.9	6.8
E. angustisporum 1	2.7	2.7	3.3	4.1	3.0	6.6
E. cassiopes 1	2.9	3.0	3.4	3.0	3.1	6.6
E. myrtilli, "myrt". 4	2.5	2.6	3.5	3.1	3.0	6.3
E. myrtilli, "vit. id." 3	2.5	2.5	3.4	4.1	2.8	6.7

No suitable inorganic buffer with a good buffering capacity at pH 4.2 was available to replace the citrate-phosphate buffer. In spite of this, a phosphate buffer was used in the following experiment, although its best buffer capacity was at pH 2 and 7 and its pH-stabilizing effect at pH 4.2 was low.

In the preceding experiments, asparagine and ammonium tartrate had proved to be the best nitrogen sources. As it was desirable to exchange these compounds for inorganic nitrogen, different inorganic nitrogen compounds were tested.

In this experiment a modified medium 2 was used. Citrate buffer was changed to phosphate buffer ($NaH_2PO_4 \cdot H_2O$ 4.1 g/l; pH adjusted with HCl to 4.1). The following nitrogen salts were tested: NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2$-tartrate and KNO_3. The amount of each source of nitrogen that was added corresponded to 0.21 g nitrogen per l. The inoculation material was cultivated on KNO_3-medium.

From Table 3 it can be seen that the pH-shifts were large by the end of the experiment. Ammonium assimilation was accompanied by a pH- drop, while in the nitrate medium there was an increase. Ammonium nitrate was an exception. The final pH was not much changed, because both ammonium and nitrate ions were utilized.

The results (Fig. 7) can be summarized as follows: NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2$-tartrate and NH_4NO_3 were almost equivalent as nitrogen sources, and these compounds supported the best growth, with the exception of *E. cassiopes* 1 for which KNO_3 was the best nitrogen source. $NH_4H_2PO_4$ was a relatively poor source of nitrogen in this experiment. The pH-shifts probably caused the final growth on KNO_3 to be better than on the ammonium salts in some cases.

In the following carbohydrate tests NH_4NO_3 was chosen as the nitrogen source. It was used in the modified medium 2, called medium 3 (p. 13).

Morton (1956), in his studies of enzyme activity in mycelia of mould fungi, found that ammonium inhibited the nitrate-reducing enzyme system. This im-

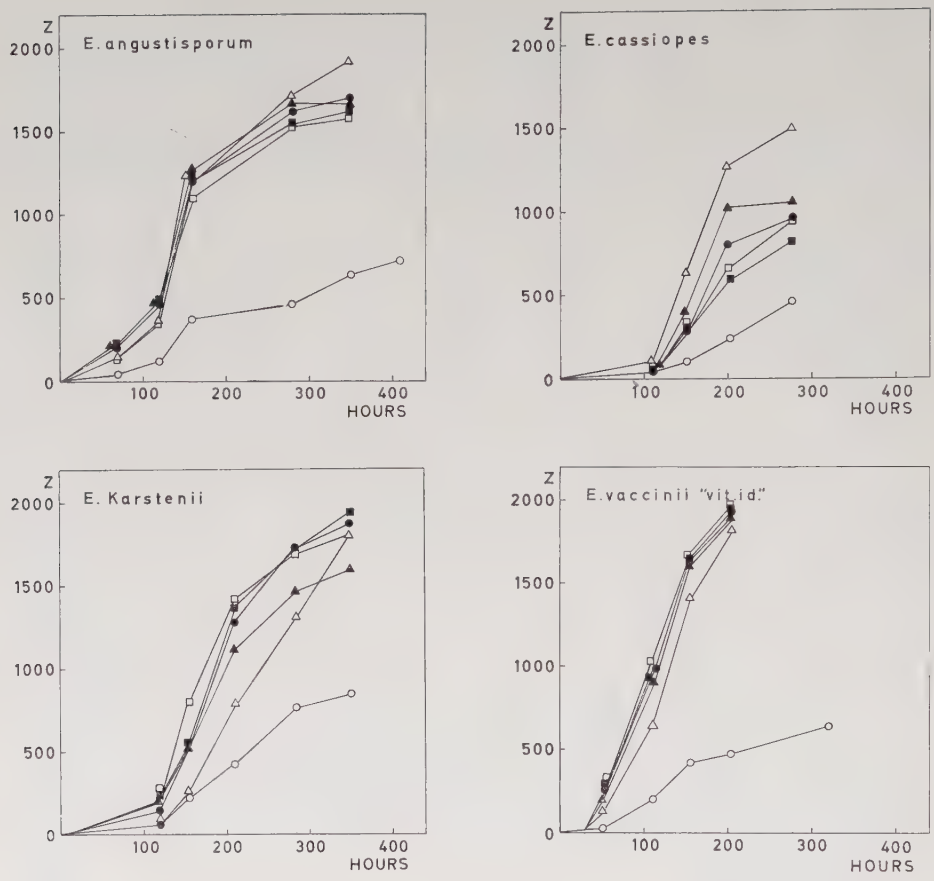


Fig. 7. The growth (measured turbidimetrically) of four species of *Exobasidium* on different nitrogen sources. —△— = KNO_3; —○— = $NH_4H_2PO_4$; —□— = NH_4Cl; —▲— = $(NH_4)_2$-tartrate; —●— = NH_4NO_3; —■— = $(NH_4)_2SO_4$.

plied that nitrate assimilation was stopped and that ammonium was utilized first when such an organism was cultivated on NH_4NO_3-medium. Not until all the ammonium was consumed did nitrate assimilation start. It ought to be possible to see preferential utilization of the ammonium ion as a pH-change. A comparison between growth and pH shift was carried out with *E. vaccinii* "vit.id." and *E. cassiopes* 1, of which the former always grew best on ammonium nitrogen and the latter on nitrate nitrogen. These two strains were cultivated on medium 3 with NH_4NO_3 or with this salt replaced by NH_4Cl or KNO_3. In each of the three series eight test-tubes were used. The growth and pH-values from this experiment are shown in Fig. 8. As the growth increased there was a continuous drop in pH of the NH_4Cl medium and a regular pH increase in the KNO_3 medium. During

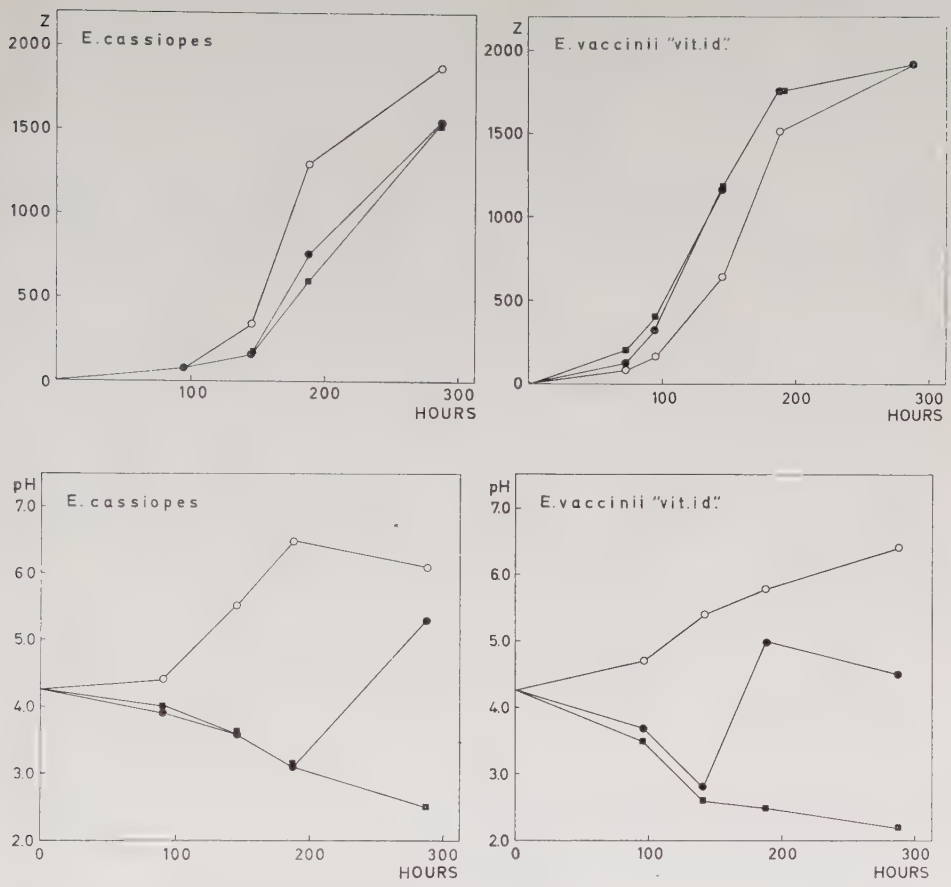


Fig. 8. The growth (measured turbidimetrically) of *E. cassiopes* 1 and *E. vaccinii* "vit. id." 1 cultured on nitrate and ammonium sources of nitrogen and the changes in pH of the media. —○— = KNO_3; —●— = NH_4NO_3; —■— = NH_4Cl.

the early stages of growth, before the culture reached a density of Z = 800–1000, the pH-shift in the medium with NH_4NO_3 was strikingly similar to that in the medium with NH_4Cl; after this Z-value had been attained the agreement of pH between the two media ceased abruptly. The drop in pH of the NH_4NO_3 medium passed over into a pH increase. This result indicates that there is preferential utilization of the ammonium ion by *Exobasidium*. No analytical studies have been made.

E. Requirements for growth substances

1. The effect of thiamine, vitamin mixture, yeast extract and N-Z case on growth

In only a few investigations has *Exobasidium* been cultivated on a synthetic medium, and the information about the vitamin requirements of this fungus is meagre. Göttgens (1959) showed that when tomato juice or the vitamins B_1, B_2, C and niacin were added, only the juice stimulated the growth of *E. azaleae* Peck (*E. japonicum*). In most experiments Göttgens cultivated this species on a synthetic medium without vitamins. Hirata (1957) cultivated 15 species of *Exobasidium*, Wolf & Wolf (1952) and Lockhart (1958) one species each on Czapek's medium. None of them mentioned that they added vitamins, and therefore these species are probably also vitamin-autotrophic.

As the present investigation also included other species, it was of interest to see how they responded to growth substances. Hence, representatives of the different *Exobasidium* host-races were cultivated on the following media:

1. Medium 2 + Difco yeast extract 200 mg per l (1)
2. Medium 2 + Difco yeast extract 200 mg per l + N–Z case 400 mg per l (a Sheffield chemical: pancreatic digest of casein) (2)
3. Medium 2 (B_1)
4. Medium 2 without thiamine (B0)

The results of these experiments can be summarized in the following manner:

1. Some strains were vitamin-autotrophic
2. Most strains required thiamine for good growth
3. The growth rate always increased when yeast extract was added but the final yield was not greater (Fig. 9)
4. N–Z case in addition to yeast extract did not further increase the growth rate.

As yeast extract was growth-promoting a series of experiments was done to find out whether a vitamin mixture has the same stimulating effect. In these experiments the following media which had optimal thiamine conc., were used:

1. Medium 2
2. Medium 2 + yeast extract 200 mg/l
3. Medium 2 + vitamin mixture (p. 14)

The yeast and vitamin mixtures were sterilized by filtering. Most of the strains were tested but no positive effect of the addition of vitamins was observed. Thus it is not probable that it was the vitamins in the yeast extract that improved

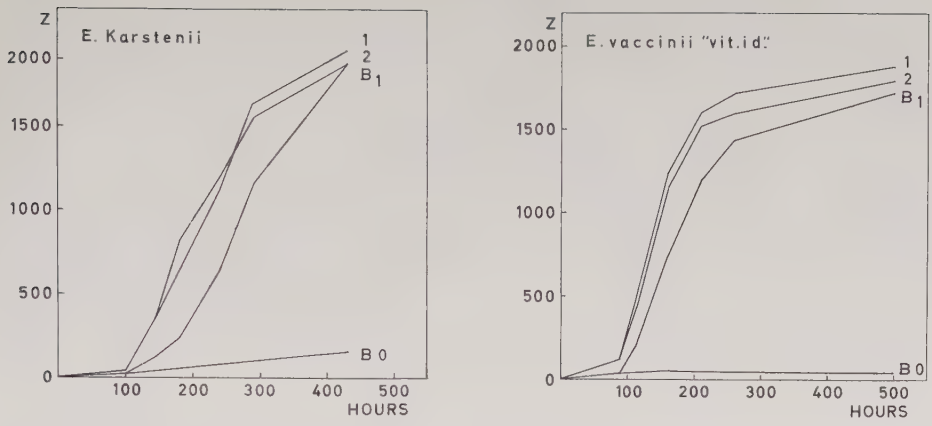


Fig. 9. The growth of *E. Karstenii* and *E. vaccinii* "vit. id." (measured turbidimetrically) in the presence of different growth substances. B 0 = no vitamins; B_1 = thiamine (0.1 mg/l); 1 = thiamine (0.1 mg/l) + yeast extract (200 mg/l); 2 = thiamine (0.1 mg/l) + yeast extract (200 mg/l) + N-Z case (400 mg/l).

the growth of *Exobasidium*. The only vitamin that the investigated vitamin-heterotrophic *Exobasidium* strains required was thiamine.

To estimate the quantitative need for this vitamin, *E. vaccinii* "vit.id.", a thiamine-requiring host-race, was cultivated in medium 2 with different concentrations of the vitamin. Thiamine was sterilized by autoclaving in solution A. In such an acid environment the vitamin is not destroyed by heating (Methods of vitamin assay). Before inoculation the conidia were washed by centrifuging.

As can be seen from Fig. 10, as low a concentration as 5 μg thiamine per l gave almost optimal growth. In the standard media 100 μg per l was used.

2. THE EFFECT OF THIAMINE AND ITS COMPONENTS

There is general agreement that metabolic deficiencies have arisen in the evolution of microorganisms by loss of an ability originally present. It is known that mutations occur by chance and that they can block metabolic activities, e.g., vitamin synthesis. There are also many examples known of how vitamin syntheses vary among different strains of the same genus or species. An extreme example of this is shown by Schulz and Atkin's (1947) investigation on *Saccharomyces cereviseae*. These authors divided the species into 13 races, all with different vitamin requirements. More examples of species with strains differing in their vitamin requirements are to be found in a review by Fries (1961 p. 337). This variation in vitamin requirement includes thiamine. Melin and Nyman (1941) studied two strains of *Boletus granulatus* and showed that one of them grew without thiamine while the other required the two components of the thiamine molecule

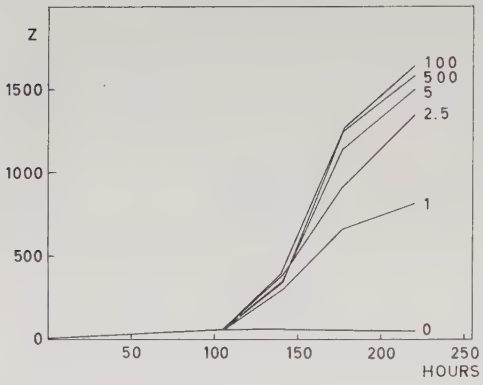


Fig. 10. The growth response (measured turbidimetrically) of *E. vaccinii* "vit. id." 1 to thiamine at 0; 1; 2.5; 5; 100 and 500 μg thiamine per l.

for good growth (thiazole and pyrimidine). Because of these examples of variation within the species, it does not appear to be advisable to use the differences in vitamin requirement as a taxonomic character, and it is natural that these characters have not been much used for classification.

However, some results indicate that some species have a typical and stable ability or lack of ability to synthesize vitamins. Swartz and Georg (1955) investigated the vitamin requirements of 50 strains of *Trichophyton tonsurans*. All of them needed thiamine (the pyrimidine component). Four cultures from two other *Trichophyton* species were tested at the same time, but these were all vitamin-autotrophic. Great stability in the thiamine requirement was also found in some *Ustilago* species, although the fungi showed differences in sporidium size, colony appearance and choice of host (Blumer 1940). Out of 215 tested strains of *Ustilago violacea* all isolates grew well in a synthetic medium supplemented with thiamine or with both thiazole and pyrimidine. Neither of the components added alone supported growth.

The interspecific variation in vitamin requirement in fungi is remarkable. Some species, e.g., certain yeasts, require five or six vitamins in the media. Almost all the studied fungi of *Agaricales*, however, require only thiamine, which in most cases can be replaced by pyrimidine. It is of taxonomic interest to see if the different *Exobasidium* species have differential ability to synthesize thiamine.

My first experiments with growth substances showed that some *Exobasidium* strains were vitamin-autotrophic while others required thiamine for optimal growth. In these experiments, only one isolate from each host-race was tested. In subsequent experiments the requirement for thiamine was tested in all isolates. The ability of the strains to synthesize the whole vitamin molecule, or its components, or to combine the latter into thiamine was investigated. Medium 2 with or without thiamine was used. The following concentrations per litre were tested:

100 μg thiamine

42.5 μg 4-methyl-5-oxyethylthiazole = thiazole

62.6 μg 2-methyl-4-amino-5-aminomethyl-pyrimidine · 2HCl = pyrimidine
42.5 μg thiazole + 62.6 μg pyrimidine
No addition.

As it was important to find out the full synthetizing capacity, even very slow synthesis must be taken into consideration. The duration of the experiments was therefore extended to 900–1000 hours. It turned out, however, that even this was not long enough, because, in some strains, thiamine synthesis started just at the end of the cultivation period. Hence, the incubation period was extended to 1500 hours in the later tests.

The minimum vitamin requirements of the *Exobasidium* strains under the prevailing conditions can be seen in Fig. 16 and may be summarized as follows:

A. Thiamine-heterotrophic strains

1. Requiring thiamine or both thiazole and pyrimidine
   E. angustisporum 1–3
   E. vaccinii "vit.id." 1–18
   E. vexans 1–5

2. Requiring pyrimidine
   E. Karstenii 4–6
   E. rhododendri 1
   E. vaccinii "Arct." 1
   E. vaccinii "myrt." 1 (after 1000 hours)
   E. vaccinii "oxyc." 1–4
   E. vaccinii "ulig." 1–6

B. Thiamine-autotrophic strains

1. With slow synthesis
   E. Karstenii 1–3
   E. myrtilli "Andr." 1, 2
   E. myrtilli "myrt." 1–5
   E. myrtilli "vit.id." 5
   E. myrtilli "ulig." 1
   E. vaccinii "Arct." 2–5
   E. uvae ursi 1, 2

With rapid synthesis
   E. cassiopes 1, 2
   E. japonicum 1–4
   E. myrtilli "vit.id." 1–4

The mixture of thiazole and pyrimidine was as growth-promoting as thiamine itself. Before the taxonomic value of the thiamine test is assessed some peculiarities of the results have to be further elucidated.

In papers by Camiener and Brown (1960) on cell-free extracts of baker's yeast and by Leder (1959), among others, everything points to the fact that the biosynthesis of thiamine and thiamine pyrophosphate proceeds by the following sequence of reactions:

$$\text{Hydroxymethylpyrimidine} \xrightarrow[Mg^{++}]{ATP} \text{Hydroxymethylpyrimidine} - P \xrightarrow{ATP}$$

$$\text{Hydroxymethylpyrimidine} - PP$$

$$\text{Thiazole} \xrightarrow[Mg^{++}]{ATP} \text{Thiazole} - P$$

$$\text{Thiazole} - P + \text{Hydroxymethylpyrimidine} - PP \xrightarrow[Mg^{++}]{} \text{Thiamine} - P + PPi$$

$$\text{Thiamine-P} \xrightarrow{H_2O} \text{Thiamine} + Pi$$

$$\text{Thiamine} \xrightarrow{ATP} \text{Thiaminepyrophosphate}$$

The pyrophosphate of vitamin B_1 is a co-enzyme that takes part in many important processes.

Knowledge of the biogenesis of the two moieties of thiamine is incomplete. It is doubtful whether the pyrimidine component of thiamine is synthesized by the same carbamyl-aspartic acid-orotic acid pathway as the pyrimidines found in nucleic acids. A recent investigation of *Escherichia coli* (Goldstein and Brown 1963) showed that the pyrimidine synthesis of thiamine did not follow this pathway.

An alternative pathway for thiamine synthesis in *Neurospora* has been proposed by Harris (1955). He found mutants of *Neurospora* which could not grow on thiazole medium, although they synthesized and accumulated pyrimidine and could grow on a mixture of thiazole and pyrimidine. He supposed that pyrimidine combined with a precursor of thiazole and produced an intermediate which was later changed to thiamine. However, the mutant could grow on thiazole as the only thiamine component if it was added in large quantities (5 μ moles thiazole/g dry weight of mycelium).

In my experiments all the strains grew as well on a mixture of thiazole and pyrimidine as on thiamine. This shows that the components are rapidly utilized by the heterotrophic strains. For strains where the mixture of thiazole and pyrimidine but not thiazole alone promoted growth, very large doses of thiazole were used (4.25 mg/l and 42.5 mg/l) in some further experiments, but there was still no growth. The pathway of thiamine synthesis proposed by Harris is not very probable and therefore I assume that the first of the pathways listed above is followed.

As the mixture of the thiamine components gave good growth, the metabolic deficiencies must be in the synthesis of these components.

3. ADAPTIVE THIAMINE SYNTHESIS

In *E. vaccinii* "vit.id." and "myrt." there were irregularities in the thiamine synthesis which are not included in Fig. 16, either because they were rather rare or because they occurred after more than 1500 hours. In the first case, when the thiamine synthesis started, it took place in only one tube out of three. These irregularities have appeared in several isolates both on pyrimidine and thiazole media and on media with no thiamine components.

This chance synthesis of thiamine in *E. vaccinii* "vit.id." and the sudden start

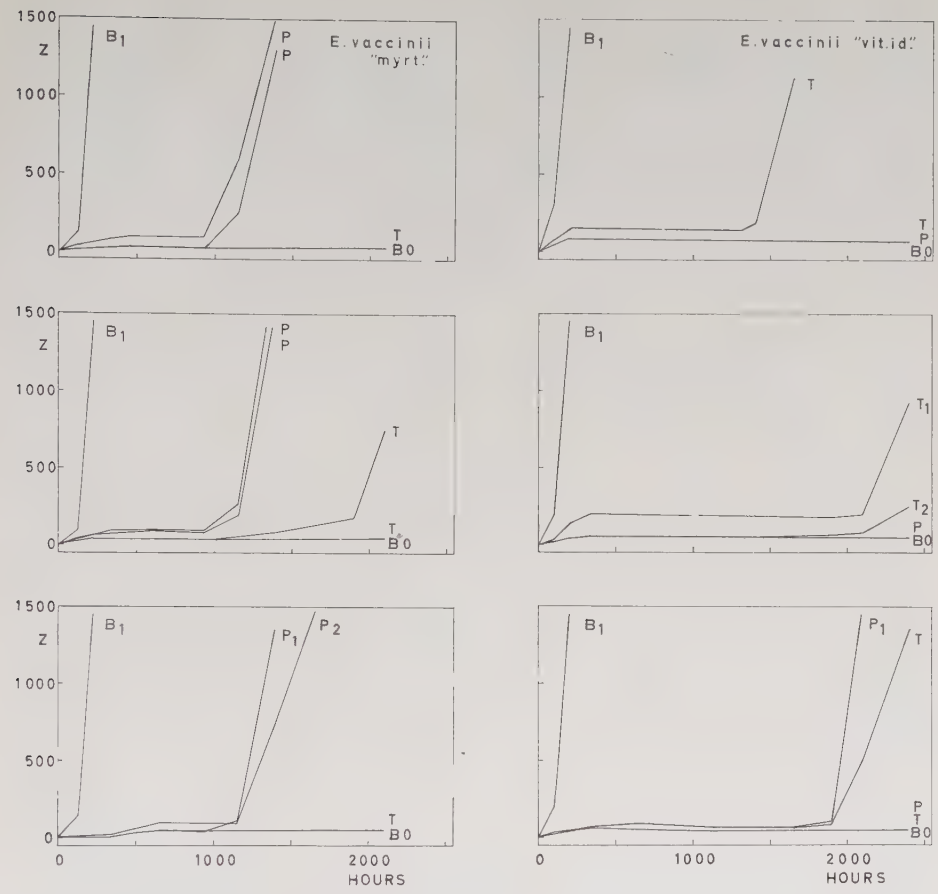


Fig. 11. The effect of thiamine and its components on the growth of *E. vaccinii* "myrt." 1 and *E. vaccinii* "vit. id." 2 on three different media:
Top: medium 2 + ribose + fructose + glycerol + succinic acid and N–Z case; middle: same medium without N–Z case; bottom: medium 2. B_1 = thiamine, T = thiazole, P = pyrimidine, B 0 = without vitamin. The treatments were duplicated and the growth values from both tubes are plotted when the growth differed. The subscripts denote cultures from which single-spore isolations were made, see also Fig. 12.

of synthesis in *E. vaccinii* "myrt." after a long lag period of 1000 hours (Fig. 16) led to a more thorough study of the thiamine synthesis of these two host-races.

Fig. 11 shows the characteristic behaviour of *E. vaccinii* "vit.id." and *E. vaccinii* "myrt.", growing on thiamine-deficient media. The fungi were cultured on three different media:

1. Medium 2 + ribose + fructose + glycerol + succinic acid + N–Z case
2. Medium 2 + ribose + fructose + glycerol + succinic acid
3. Medium 2

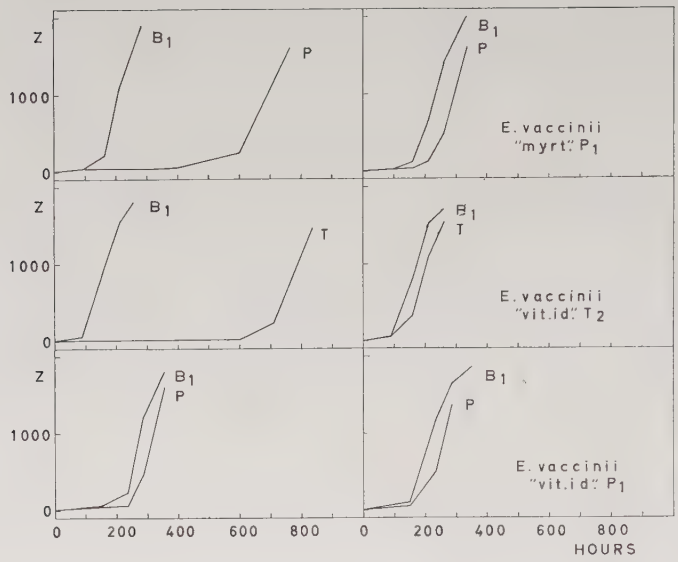


Fig. 12. The influence of deadaptation, by culturing on thiamine-containing media, of *E. vaccinii* "myrt." 1 and *E. vaccinii* "vit. id." 2 adapted to grow on pyrimidine- or thiazole-containing media, on the ability subsequently to utilize pyrimidine or thiazole. Left side: replicates subcultured ten times on thiamine-containing media; right side: replicates subcultured on the media to which the adaptation had occurred. Figures on the same level represent the same strain after (left) and before (right) the deadaptation process.

For each medium the growth on thiamine, thiazole, pyrimidine or on no thiamine components was tested. On all three media, the growth of *E. vaccinii* "myrt." always started after about 1000 hours in the series with pyrimidine (Fig. 11). On the other hand, *E. vaccinii* "vit.id." was much more irregular in its growth reactions. To investigate which processes regulated the adaptive growth, monospore cultures were isolated from the adapted strains (T_1, T_2 and P_1 from *E. vaccinii* "vit.id." and P_1 and P_2 from *E. vaccinii* "myrt." in Fig. 11). Parallel series of these monospore cultures were grown for five weeks on thiamine medium, and on the medium on which the adaptation had occurred. During this period the strains were transferred ten times to new media. After this, the ability of the two replicates to grow on the medium on which the adaptation had occurred was tested. All strains that had been cultured on the adaptation media started growth on this medium almost as soon as on thiamine medium. This good growth could already be seen by the first transfer. The parallel cultures pre-grown on thiamine medium behaved differently when they were transferred back to the adaptation media. Deadaptation had occurred in the two isolates of *E. vaccinii* "myrt." (P_1 and P_2) and the thiazole isolates of *E. vaccinii* "vit.id." (T_1 and T_2) while a pyrimidine isolate of the latter (P_1) showed no deadaptation. In Fig.

12 only the results for *E. vaccinii* "myrt." P_1 and *E. vaccinii* "vit.id." T_2 and P_1 are represented. The deadaptation of the cultures and other results presented below indicate that enzymatic adaptation had occurred in *E. vaccinii* "myrt." on pyrimidine medium and in *E. vaccinii* "vit.id." on thiazole medium. However, in *E. vaccinii* "vit.id." on pyrimidine a selection of a mutant was the probable cause of the adaptive growth. It is difficult to decide by genetical analyses if there are any genetic differences between the strains before and after the adaptation, because *Exobasidium* does not cross outside the host, and because it is homothallic.

The results of the test indicated that two different processes were responsible for the adaptive growth, a mutational one (genotypic adaptation) that appeared irregularly, and an enzymatic adaptation that was remarkably constant (phenotypic adaptation).

Further experiments on medium 2 have shown that in *E. myrtilli* "myrt." enzymatic adaptation appeared after 1000 hours in pyrimidine series, while it usually was delayed until about 2000 hours on media with thiazole or without thiamine components. The adaptation of *E. vaccinii* "vit.id." usually took more than 2000 hours, and there were only small differences in rate of adaptation on media with and without thiamine components. Examples of adaptation on agar can be seen in Photo 2.

It appears from Ryan's work (1955) that spontaneous mutations can arise in the stationary growth phase in both liquid and agar media; this might explain the appearance of the rare mutations after such a long time in the *Exobasidium* experiments.

The non-mutational adaptations of *Exobasidium* are difficult to explain. Yates and Pardee (1957) showed that the synthesis of orotic acid, a metabolic precursor of the pyrimidines in the nucleic acid, could be inhibited by uracil. They found that uracil repressed the enzymes that catalyzed the synthesis of orotic acid much more than did orotic acid. In my investigation the fungi have been cultivated on thiamine medium, and it might be possible that thiamine represses the enzymes for the biosynthesis of thiamine, so that when the fungus was put into a medium without thiamine, enzymes for thiamine synthesis had to be built up.

Whether or not there is a repression before the adaptation in the thiamine-free medium, it took an extremely long time for the *Exobasidium* strains to start growth. It is also possible that there was a different balance or a synthesis of new metabolites, when the fungus was in the stationary phase, which influenced the thiamine synthesis.

In another series of experiments the adaptive conditions were intended to be more favourable. The reason for one of the experiments arose from an observation of Kramer and Straub (1957) who came to the conclusion that *Bacillus cereus* induced penicillinase when the synthesis for an RNA specific for penicillinase

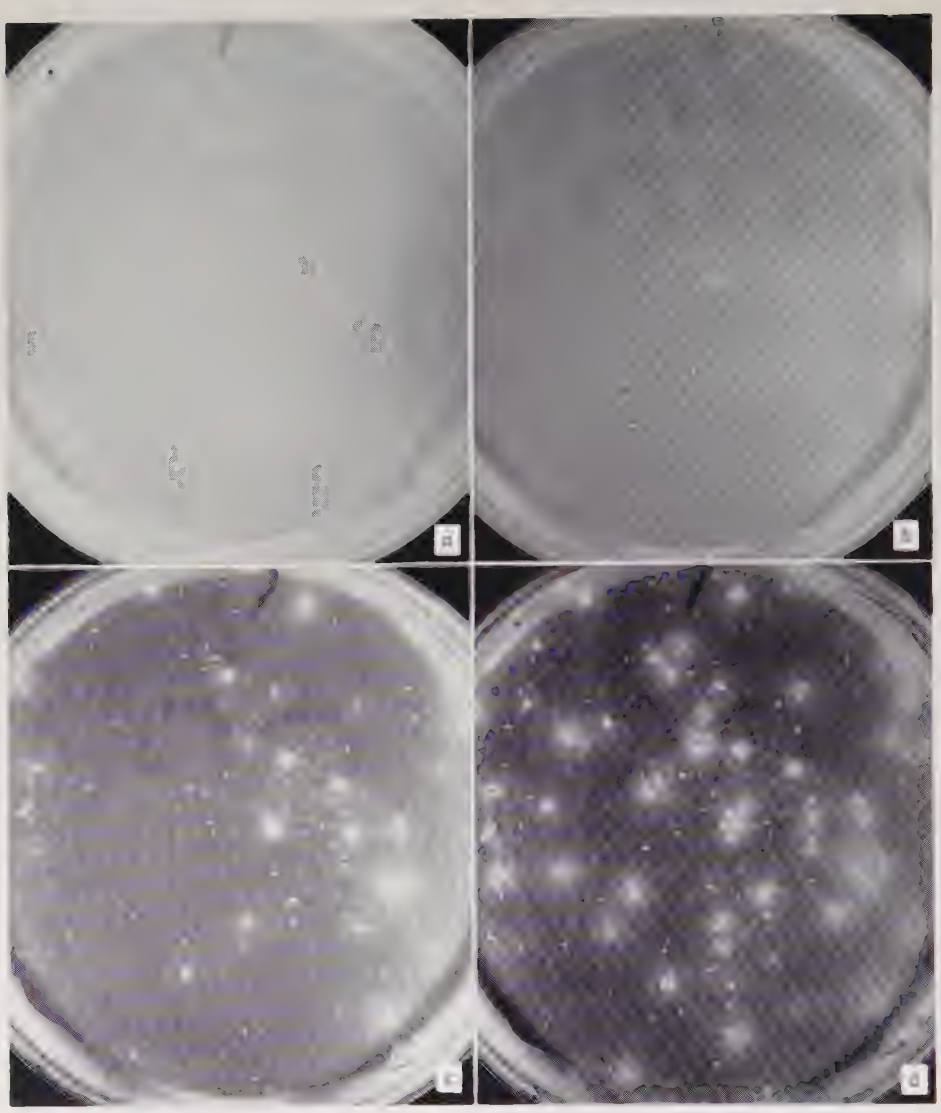


Photo 2. Adaptation to growth of *E. vaccinii* Myrt. The moculum was mixed in the molten agar medium 3 without thiamine at pH 5.0. Incubation time: a = 15 days; b = 65 days; c = 85 days; d = 105 days. 10^5 conidia/plate.

production was favoured by addition of adenine, guanine, cytosine and uracil. For this organism the same result was obtained when it was starved before the induction. To investigate whether there is competition between the ribonucleic acids active in the reproduction of the proteins of the cell and in the production of the enzymes catalyzing the thiamine synthesis for the pyrimidines and

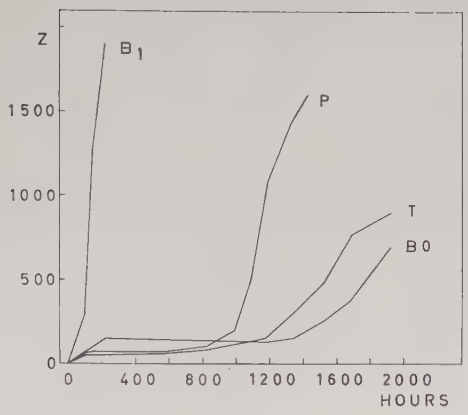


Fig. 13.

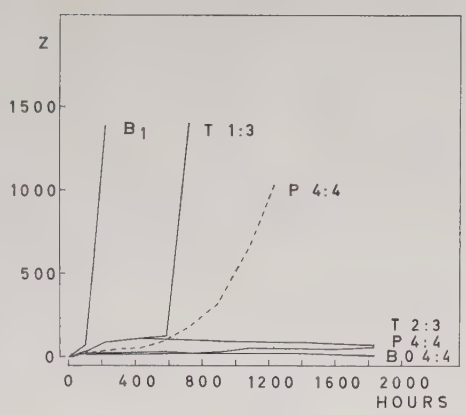


Fig. 14.

Fig. 13. Growth (measured turbidimetrically) of *E. vaccinii* "myrt." 1 on medium 1 with thiamine or its components. Initial pH = 4.0. B_1 = thiamine, T = thiazole, P = pyrimidine, B 0 = no vitamin.

Fig. 14. Growth (measured turbidimetrically) of *E. vaccinii* "vit. id." 2 on medium 1 with thiamine or its components. Unbroken line = initial pH 4.0; broken line = initial pH 3.0. The numbers by the curves indicate the number of tubes represented by the curves, and the total number of tubes.

purines of the ribonucleic acid, *E. vaccinii* "vit.id." and *E. vaccinii* "myrt." were cultivated on medium 2 with a mixture of adenine, guanine, cytosine and uracil added. These bases (1 μ M sterilized by filtering) did not affect the induction of the thiamine synthesis. The experiment also showed that the pyrimidines of the nucleic acids could not replace the pyrimidine of thiamine and were not converted into its precursors. Replacing glucose by the more slowly utilized ribose also had no effect on the thiamine synthesis. Robbins and Kavanagh (1938) showed that the thiamine requirement can appear at high salt concentrations. However, a tenfold dilution of medium 2 did not change the thiamine requirement in *Exobasidium*. When medium 2 was exchanged for medium 3 and 1, the lag phase in *E. vaccinii* "myrt." was somewhat shortened, while *E. vaccinii* "vit.id." was not affected. The influence of pH on thiamine synthesis was also studied. At pH 3.0, 4.0, 5.0 or 6.0 on medium 1, *E. vaccinii* "myrt." had about the same synthetizing capacity. On medium 1 this host-race regularly showed an adaptation to growth, in all tubes with one or none of the thiamine components (Fig. 13).

On the other hand, *E. vaccinii* "vit.id." was clearly stimulated to growth on the pyrimidine medium at pH 3.0. The growth started at the same time in all four tubes, thus indicating an enzyme induction. At the higher pHs, there was no growth on pyrimidine media (Table 4 and Fig. 14). Growth of a mutational

Table 4. Growth of *E. vaccinii* "vit. id." 2 on thiamine and its components at pH 3.0 and 4.0.

The growth was measured turbidimetrically and is represented by *Z*-values at different times.

	Time in hours											
	98	146	220	435	580	720	890	1085	1230	1420	1590	1830
						No thiamine						
pH 3.0	30	40	40	50	90	90	140	180	190	780	1310	1600
	45	80	90	90	120	120	110	130	130	130	110	110
	20	50	70	70	90	80	80	90	80	80	65	70
						Thiazole						
	30	60	80	80	140	90	70	100	125	110	70	60
	20	50	60	40	50	40	40	50	40	40	40	30
	10	20	25	25	50	40	30	50	50	50	30	30
						Pyrimidine						
	15	20	10	30	80	190	350	630				
	10	15	10	30	70	120	220	640	1600	1700	1900	1700
	15	30	40	60	120	200	360	700	830	950	1100	1250
	30	45	70	100	140	190	300	610	630	720	810	990
						Thiamine						
	60	470	1390									
	30	355	1310									
						No Thiamine						
pH 4.0	20	30	25	20	40	20	20	30	25	20	0	0
	20	20	20	30	40	20	25	25	30	20	0	40
	25	20	30	40	50	25	35	30	35	30	0	0
	10	25	15	10	20	15	10	15	15	10	0	0
						Thiazole						
	40	70	90	130	130	110	100	100	110	100	100	100
	20	40	70	90	110	80	70	70	70	70	40	40
	25	50	110	125	130	1400	1800					
						Pyrimidine						
	10	10	10	10	25	25	20	50	70	50	25	20
	25	20	20	15	25	25	40	40	35	40	45	60
	25	20	20	20	30	30	30	90	90	50	30	40
	30	30	30	20	40	30	50	60	50	70	100	130
						Thiamine						
	60	590	1350									
	80	640	1420									

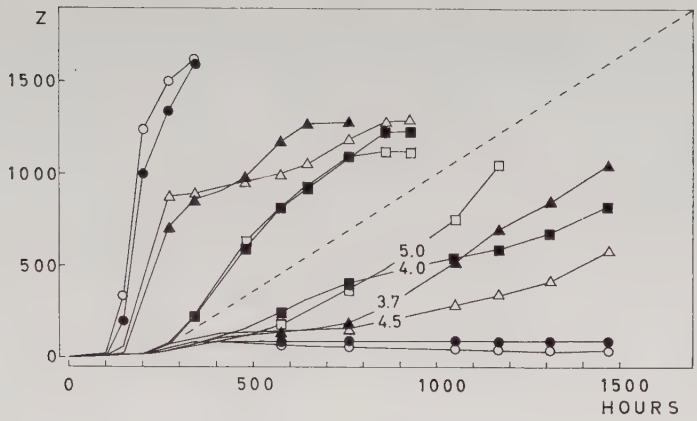


Fig. 15. The growth (measured turbidimetrically) of *E. vaccinii* "vit. id." 2 on medium 3 with thiamine or pyrimidine with different carbon sources and pH.

Glucose, pH 4.2 = ♂; oxalacetic acid, pH 4.2 = □
Glucose, pH 3.4 = ●; oxalacetic acid, pH 3.4 = ■
Oxalacetic acid + α-ketoglutaric acid, pH 4.2 = △
Oxalacetic acid + α-ketoglutaric acid, pH 3.4 = ▲

Growth curves to the right and left of the broken line are for pyrimidine- and thiamine-media, respectively.

type occurred in one tube out of three on the thiazole medium at pH 4.0 and in one tube without thiamine components at pH 3.0 (Table 4). Growth on media without thiamine components or with thiazole was not influenced by pH.

In a "by-passing" experiment using medium 3 with α-ketoglutaric acid + oxalacetic acid or oxalacetic acid as carbon sources instead of glucose, adaptation to growth occurred on the pyrimidine medium in *E. vaccinii* "vit.id.". In this experiment, the initial pH for the two replacement carbon sources and the control with glucose was 4.2 or 3.4. As can be seen from Fig. 15, both oxalacetic acid + α-ketoglutaric acid and oxalacetic acid alone supported better growth on the pyrimidine media than the control with glucose. Although *E. vaccinii* "vit.id." had a somewhat better growth at the higher pH in thiamine media, growth started earlier at the lower pH in pyrimidine media. In contrast to the results shown in Fig. 14, the low pH did not stimulate growth on the glucose-pyrimidine medium. Probably pH 3.4 was too high for growth to start. It is also noticeable that oxalacetic acid stimulated the growth more than the mixture of oxalacetic acid + α-ketoglutaric acid. Replacement of glucose and the change of pH had no influence on the growth in thiazole media or media without thiamine components.

As thiamine is active as co-enzyme in many important reactions, its absence blocks many sequences of reactions. For example, it is known that low levels of

this vitamin cause accumulation of puruvate. Hypothetically, it is possible that the accumulated compound represses the enzymes that have catalyzed the formation of the compounds, but also that it induces enzymes which attack the accumulated substrate. The normal pathway via the Krebs cycle is blocked by the absence of thiamine, but other pathways with enzymatic reactions catalyzed by enzymes without thiamine can be induced. The growth on oxalacetic acid of *E. vaccinii* "vit.id." seemed at first sight to support the hypothesis of an alternative pathway, but as thiamine-free media or media with thiazole did not have the same effect, the growth in pyrimidine media must depend on the synthesis of thiazole.

Investigations of adaptation to growth in thiamine-dependent strains have been made by Eberhart and Tatum (1961, 1963). They studied a mutant of *Neurospora crassa*, which, unlike the wild type, required thiamine for immediate growth. Depending on the amount of the thiamine in the medium, the growth of the mutant started 1–2 days after the wild type. Other thiamine-less mutants, one lacking thiazole-synthesizing capacity, the other the power to condense thiazole and pyrimidine, also adapted to growth on minimal medium, but only after more than one week's incubation. The progeny of the crosses between the two latter mutants did not adapt.

The authors found no differences between the wild type and the first-mentioned mutant on analysis of the thiamine-active substances. They showed that the two types diverged in the utilization of thiamine-phosphates. The biochemical basis of the adaptation in the other mutants has not been studied.

The series of thiamine experiments have shown that the studied host-races had a stable and characteristic synthesizing capacity. In some cases there was adaptation to thiamine synthesis after a long time. This adaptation can be caused by mutations or by enzymatic inductions. The former (genotypic adaptation) appears irregularly, usually at different times and in only a few tubes of the series. The frequency varies in different host-races. The thiamine mutations have been observed only in *E. vaccinii*. The occurrence of enzyme induction (phenotypic adaptation) was to some extent dependent on the environment and the nutritional conditions. To be comparable, vitamin tests must be made under exactly the same conditions.

F. The utilization of carbohydrates

The ability to utilize different sources of carbon has often been used for the characterization of species of fungi and in some cases also of genera. Even among pathogenic fungi these tests have been used. Mix (1954) studied 26 species of *Taphrina* and all of them possessed a characteristic pattern of utilization of the various carbohydrates. Within a single species different isolates isolated from the

same host species agreed nutritionally, while isolates from different host species showed a tendency to differ in their physiology.

In earlier experiments with *Exobasidium* (Göttgens 1959) only glucose and sucrose have been used. In the following assimilation tests the compounds that Wickerham and Burton (1948) had proposed were used. These authors tested some 70 compounds, and those carbohydrates which in their investigation were the most useful in differentiating species were also used in the following investigation. Besides these compounds, the glucosides, arbutin and salicin, were tested. Arbutin and methyl-arbutin are stored in large quantities in the members of *Ericaceae*. Tests made by Hemberg (1952) showed that leaves of *Arctostaphylos uva-ursi* contained 13 per cent arbutin on a dry weight basis. Racz, Fuzi, and Kisgyorgy (1961) found 2.7–6.7 % arbutin in the leaves of *Vaccinium vitis-idaea*. Arbutin has previously been used in taxonomic studies of yeast (Cook 1958 p. 280). It is hydrolyzed by β-glucosidase to form glucose and hydroquinone. Barnett *et al.* (1956) studied the activity of β-glucosidase on 119 strains of yeast and suggested that there was some specificity for it. Strains that hydrolyzed arbutin did not always split other β-glucosides, and Barnett *et al.* suggested that it would be worth while to utilize arbutin, salicin, and cellobiose for classification purposes.

In the first carbohydrate test, 33 strains representing 16 host-races were tested for their capacity to utilize the 25 different compounds listed below:

Pentoses:	D-arabinose, L-arabinose, D-ribose, D-xylose
Hexoses:	D-galactose, glucose, sorbose, rhamnose
Disaccharides:	cellobiose: β-glucosido-glucose
	lactose: β-galactosido-glucose
	melibiose: α-galactosido-glucose
	maltose: α-glucosido-glucose
	sucrose: α-glucosido-β-fructofuranosid
Trisaccharide:	raffinose: α-galactosido-α-glucosido-β-fructofuranosid
Sugar alcohols:	erythritol, dulcitol, mannitol, sorbitol
Polysaccharides:	starch, inulin
Organic acids:	citric acid, lactic acid, succinic acid
Glycosides:	arbutin: hydroquinone-β-glucopyranoside
	salicin: saligenin-β-glucopyranosid

Medium 3 with a vitamin mixture was used, and the initial pH was 4.2. Solution A without sugar and with half the quantity of water was autoclaved separately. The carbohydrates were dissolved in the other half of the water. The amounts of the different carbohydrates were calculated to correspond to the quantity of carbon in 2 per cent glucose solution. Raffinose and starch were tested at only half this concentration. Arbutin was added in both these amounts. The polysaccharides were sterilized with ether; the di- and trisaccharides, glucosides, xylose and vitamin mixture were sterilized by filtration and the rest of the carbohydrates were autoclaved.

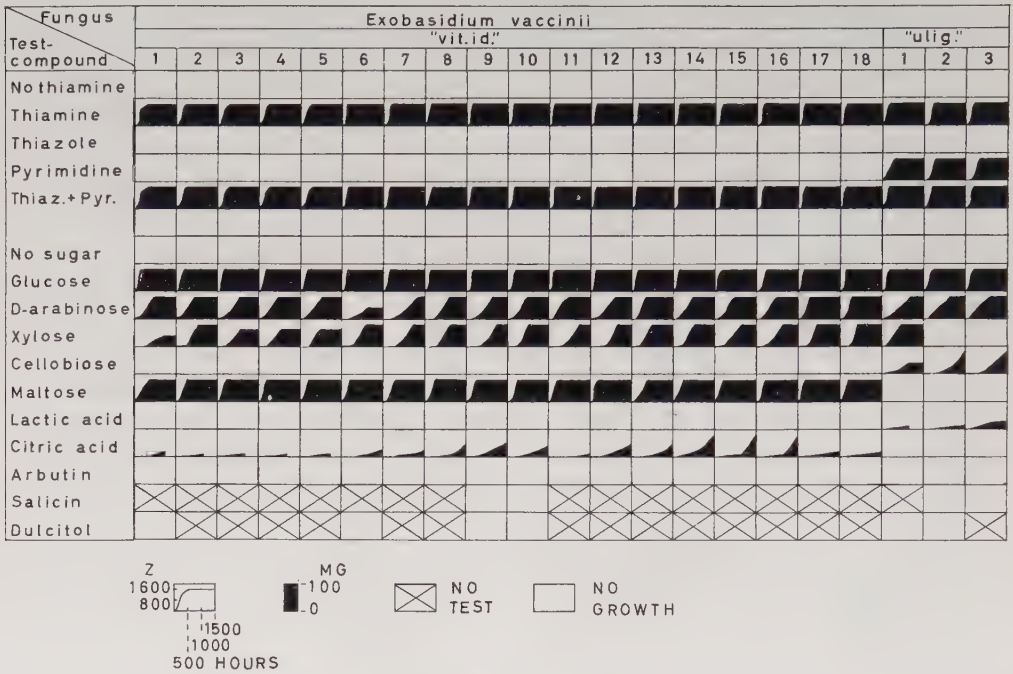

Fungus
Test-compound
Exobasidium vaccinii
"vit.id."
"ulig."
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 1 2 3
No thiamine
Thiamine
Thiazole
Pyrimidine
Thiaz.+Pyr.
No sugar
Glucose
D-arabinose
Xylose
Cellobiose
Maltose
Lactic acid
Citric acid
Arbutin
Salicin
Dulcitol
Z
1600
800
1500
1000
500 HOURS
MG
100
0
NO TEST
NO GROWTH

Fig. 16.

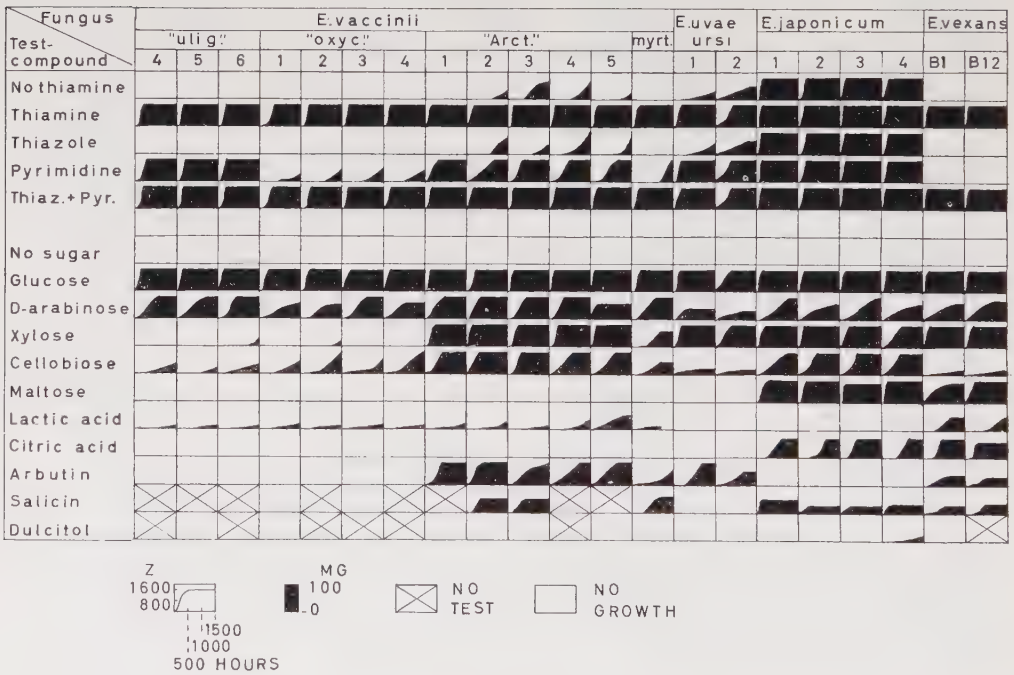

Fungus
Test-compound
E.vaccinii
"ulig." "oxyc." "Arct." myrt.
E.uvae ursi
E.japonicum
E.vexans
4 5 6 1 2 3 4 1 2 3 4 5 1 2 1 2 3 4 B1 B12
No thiamine
Thiamine
Thiazole
Pyrimidine
Thiaz.+Pyr.
No sugar
Glucose
D-arabinose
Xylose
Cellobiose
Maltose
Lactic acid
Citric acid
Arbutin
Salicin
Dulcitol
Z
1600
800
1500
1000
500 HOURS
MG
100
0
NO TEST
NO GROWTH

Fig. 16.

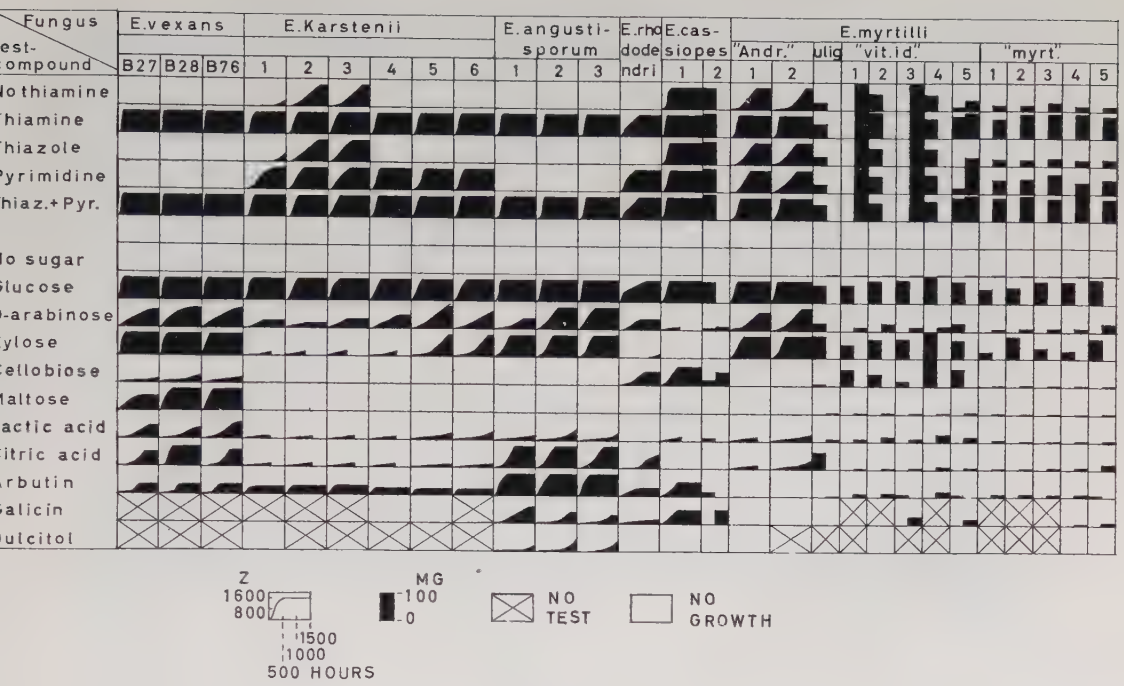


Fig. 16.

Fig. 16. The ability of *Exobasidium* strains to grow on thiamine and its components as the only vitamin, and to utilize different carbon sources. Medium 2 and 3, respectively, were used. The growth curves are based on measurements of turbidity and the columns on dry weights (mg/flask). A bar in the left of the square indicates harvest after 15–20 days; in the right after 55–65 days. The measurements of turbitity were made at least twice a week; the curves are of growth-values up to Z = 1600.

The xylose was filtered because Lockwood and Nelson (1946) stated that autoclaved xylose was broken down to furfural.

When measuring growth in the media containing polysaccharides, noninoculated tubes of media with the polysaccharides were used as controls, as these nutrient solutions were turbid and the turbidity increased during the experiment. The purpose of this carbohydrate test was chiefly to reveal physiological characters which could be used in taxonomic differentiation; the results can be summarized as follows:

1. Compounds utilized by all the tested *Exobasidium* strains (Fig. 6): D-arabinose, L-arabinose (except *E. vaccinii* "ulig." 2), D-ribose, D-galactose, glucose, sucrose, raffinose, erythritol, mannitol, sorbitol, succinic acid, (growth curves on D-arabinose can be seen in Fig. 16).

2. Compounds not utilized by any strains: rhamnose, sorbose, lactose, melibiose, inulin, and starch.

3. Compounds utilized by some groups and not utilized by others and hence of taxonomic value: D-xylose, maltose, cellobiose, dulcitol, citric acid, lactic acid, arbutin, and salicin (Fig. 16).

The compounds in groups 1 and 2 lack taxonomic interest. The degree of utilization of the tested compounds is of secondary importance and it need only be mentioned that glucose and sucrose were throughout the best carbon sources. For further information see Fig. 6 and 16.

On the other hand, a more thorough study was devoted to the compounds of group 3. All isolated strains were tested on these compounds and on glucose and D-arabinose as reference substances, since it was of interest to see whether the physiological properties found in this experiment were constant and characteristic of the studied species or host-races.

As can be seen from Fig. 16, the strains within each host-race utilized the tested compounds very uniformly, while there are obvious differences between the host-races. In only a few cases did strains belonging to the same host-race differ in the utilization of a compound. Fig. 16 shows that only a few strains of *E. vaccinii* "ulig." and *E. vaccinii* "oxyc." grew on xylose. However, when some experiments were extended to 2000 hours, all strains of *E. vaccinii* "ulig." and *E. vaccinii* "oxyc." were found to be able to assimilate xylose. In all these strains the adaptation to xylose occurred between 1200 and 2000 hours, except in *E. vaccinii* "ulig." 1 which began to grow almost at once. In *E. cassiopes* the lack of ability to utilize xylose was absolute. Dulcitol was a poor carbon source for one strain of *E. japonicum*; in another strain a mutation which caused rapid growth occurred after 800 hours in one of the test tubes. The only species where all strains made use of dulcitol was *E. angustisporum* but this was only after a long lag phase. As regards the growth on organic acids, a change of pH also altered the degree of utilization. This may be caused by changes in permeability and in the degree of dissociation of the acid. Thus, the results from the tests on utilization of acids do not necessarily reflect the real ability of the cells to metabolise them. After 800 hours, a mutation occurred in one out of six tubes of *E. vaccinii* "myrt." on citric acid medium. In *E. myrtilli* "vit.id." a mutation also occurred; this mutant is described on p. 48.

In the utilization of arbutin the medium became coloured brown, probably by the oxidation of the freed hydroquinone. Therefore the curves of growth of arbutin based on turbidimetric determinations are somewhat too high and comparisons with the curves of growth on the other compounds cannot be made. Microscopic studies and weighings showed that a correlation existed between growth on arbutin and the turbidimetric values.

In spite of the fact that arbutin showed no fungistatic effect, Siebs (1955) claimed that it probably was important for scab resistance in the leaves of the

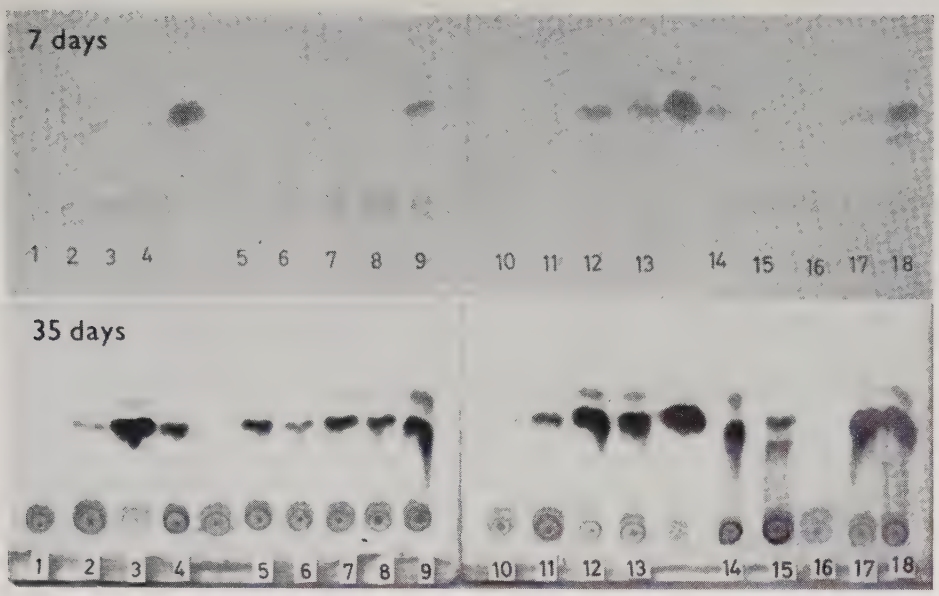


Photo 3. Thin layer chromatograms showing the arbutin-splitting capacity of *Exobasidium* strains after 7 and 35 days. Hydroquinone was run as a standard between spots 4–5 and 13–14. The numbers refer to the strains listed in Table 5.

pear-tree, as the glucoside can be enzymatically split into hydroquinone and glucose. Higher contents of hydroquinone were also found in the leaves of resistant varieties than in susceptible ones.

Hildebrand and Schroth (1963) showed that the nectaries of *Pyrus communis* had lower antibiotic activity towards *Erwinia amylovora* than the surrounding tissues and were preferentially invaded by the fungus. They suggested that the antibiotic activity depended on the differential occurrence of β-glucosidase, because chromatography and bioassays of the toxic extracts indicated that the toxic compound was hydroquinone and the content of arbutin showed no differences.

Only some of the *Exobasidium* host-races utilized arbutin. It is possible that more host-races possess enzymes that split arbutin into glucose and hydroquinone but that they were not able to grow on the free glucose, because of the accumulation of toxic hydroquinone. Hence it is of interest to follow the hydrolysis of arbutin by the different host-races and to investigate the toxity of hydroquinone.

To investigate the activity of glucosidase towards arbutin, strains of the different host-races were cultivated on medium 3 with glucose replaced by arbutin. After 7, 10, 14 and 35 days, the arbutin-splitting activity of the fungi was tested. The fungi were removed from the culture solutions by centrifugation, and after the solutions had been concentrated to the same extent, the arbutin and hydro-

Table 5. Growth measured turbidimetrically of different *Exobasidium* strains on arbutin as the only carbon source and their ability to hydrolyze arbutin to hydroquinone.

No.	Tested strain	Growth in Z-values on days				Hydroquinone on days			
		7	10	14	35	7	10	14	35
	E. vaccinii								
1	— "vit. id." 10	50	40	50	30	−	−	−	−
2	— "myrt." 1	60	60	100	280	−	+	+	+
3	— "Arct." 2	240	520	840	960[a]	+	+	+	+
4	— "Arct." 4	90	140	320	790[a]	+	+	+	+
5	— "ulig." 2	40	40	55	50	+	+	+	+
6	— "ulig." 4	20	20	30	20	+	+	+	+
7	— "oxyc." 1	20	30	30	20	+	+	+	+
8	— "oxyc." 4	10	10	30	60	−	−	+	+
9	*E. Karstenii* 4	270	410	440	380	+	+	+	+
10	*E. myrtilli* "Andr." 2	40	30	30	30	−	−	−	+
11	*E. uvae ursi* 1	50	50	100	280[a]	−	−	+	+
12	*E. vexans* B_1	170	310	760	570[a]	+	+	+	+
13	*E. japonicum* 1	140	200	175	170	+	+	+	+
	E. myrtilli								
14	— "myrt." 4	90	110	230	400[a]	+	+	+	+
15	— "vit. id." 3	70	90	200	300[a]	+	+	+	+
16	— "ulig." 1	20	20	20	10	−	−	−	
17	*E. cassiopes* 1	90	100	250	400	+	+	+	+
18	*E. angustisporum* 1	1150	1210	1500	2000[a]	+	+	+	+

+ = Splitting capacity; − = no splitting capacity.

[a] The colour of the solution after 35 days was yellowish brown.

quinone contents of the culture solutions were studied by thin layer chromatography in accordance with Stahl and Schorn (1961). Phosphomolybdic acid was used as the reagent for identification. Table 5 and Photo 3 show the result of the test.

Between spots 4–5 and 13–14 hydroquinone was run as a standard. With the used solvent (toluene—ethyl formate—formic acid), arbutin remained at the starting point. The spots above and below the hydroquinone spots, which occur in some culture solutions, have not been identified. About the same quantity of each culture solution was used. The faintest hydroquinone spot that could be detected corresponded to about 10–20 mg hydroquinone per l.

Photo 3 shows the degree of glucoside splitting after 7 and 35 days.

E. myrtilli "ulig." 1 and *E. vaccinii* "vit.id." 10 did not produce detectable amounts of hydroquinone. This absence of hydrolysis was also found with other tested strains of *E. vaccinii* "vit. id.". In *E. myrtilli* "Andr." 2 the rate of hydro-

Table 6. The tolerance of the *Exobasidium* strains towards hydroquinone.

Strains	Growth % of control at different hydroquinone concs.			
	10^{-4} *M*	10^{-3} *M*	10^{-2} *M*	$2 \cdot 10^{-2}$ *M*
E. vaccinii "vit. id." 10	100	100	60	0
E. vaccinii "Arct." 2	100	100	100	0
E. vaccinii "ulig." 2	100	80	0	0
E. vaccinii "oxyc." 1	100	30	0	0
E. Karstenii 4	100	100	20	0
E. myrtilli "Andr." 2	100	100	30	0
E. uvae ursi 1	100	100	10	0
E. japonicum 1	100	30	0	0
E. angustisporum 1	100	100	80	0
E. cassiopes 1	100	100	0	0
E. vaccinii "myrt." 1	100	100	70	0
E. vexans B_1	100	100	30	0

lysis was very low and only a very faint spot could be detected after 35 days. As can be seen in Photo 3 there was, as early as 7 days after inoculation, a rather large accumulation of hydroquinone in the culture media of *E. vaccinii* "Arct.", *E. Karstenii*, *E. vexans*, *E. japonicum*, *E. myrtilli* "myrt.", and *E. angustisporum*. The faintest spots cannot be seen in the photograph.

It is obvious from the photograph and the table that the number of strains hydrolyzing arbutin to hydroquinone was greater than the number of those that grew appreciably on it. It seemed then to be of interest to study how the fungi were able to tolerate the freed hydroquinone. The fungi were cultivated on medium 3 with hydroquinone added. The hydroquinone was sterilized by filtration and the pH of the media of 4.2 was favourable because at higher pH hydroquinone is more easily oxidized to the more toxic p- bensoquinone (Potter 1942).

The test showed that tolerance towards hydroquinone was very different among the strains (Table 6). The host-races that were least inhibited by hydroquinone also grew best on arbutin, but *E. vaccinii* "vit.id." and *E. myrtilli* "Andr.", which lacked an effective capacity to hydrolyze arbutin, tolerated high concentrations of hydroquinone. The first ten strains in Table 6 were also tested for their ability to utilize hydroquinone as the only carbon source. Medium 3 with glucose replaced by filtered hydroquinone was used. Two of the tested strains *E. vaccinii* "Arct." and *E. angustisporum* assimilated hydroquinone (Fig. 17); the others did not.

This series of experiments has shown that the growth of *Exobasidium* on arbutin is controlled by several processes. Only *E. vaccinii* "vit.id." and *E. myrtilli* "ulig." lacked arbutin-splitting glucosidase activity. Enzymatic induction occur-

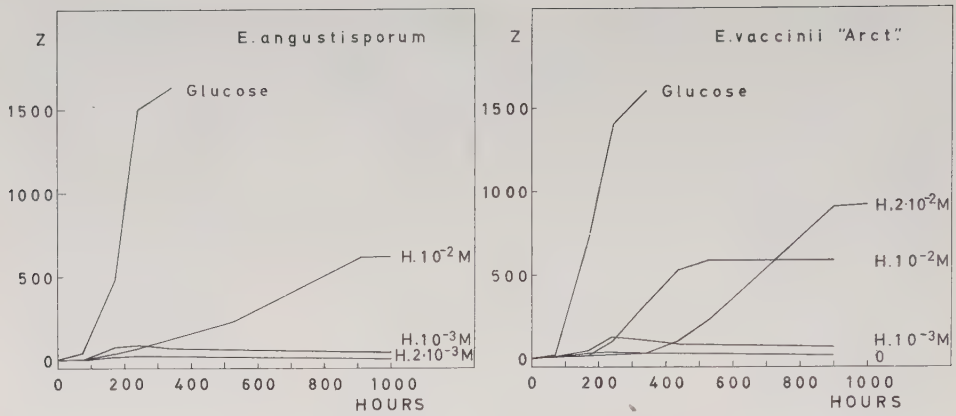


Fig. 17. The growth (measured turbidimetrically) of *E. angustisporum* 1 and *E. vaccinii* "Arct." 2 on hydroquinone as the only carbon source. The glucose of medium 3 was replaced by 10^{-2}, $2 \cdot 10^{-3}$ and 10^{-3} M hydroquinone (= H).

red in *E. vaccinii* "myrt." and in *E. uvae ursi*. The hydrolyzing activity was observed after 10 and 14 days, respectively (Table 5), and in the growth curves in Fig. 16.

Of the strains that split arbutin, *E. vaccinii* "Arct." and *E. angustisporum* were able to assimilate the freed hydroquinone. The remaining strains showed different degrees of sensitivity to hydroquinone. It seemed to be the degree of tolerance towards hydroquinone that determined the ability of these strains to utilize arbutin.

All fungal cells were coloured brown when they grew on arbutin media, except *E. vaccinii* "myrt.". In a few cases the pigment passed into the culture medium (Table 5).

On other media when growth began to cease, a brown pigment appeared in the cells of *E. myrtilli*, *E. vexans* and *E. japonicum*. The host-races differed as regards this character. In *E. myrtilli* "vit.id." much pigment was produced and the pigment diffused out into the medium, which became coloured dark brown. This pigment production was less conspicuous in *E. myrtilli* "Andr.", *E. vexans* and *E. japonicum*, and did not occur at all in *E. myrtilli* "myrt." and "ulig.". The pigment-producing host-races constantly exuded the pigment in all the media used in this investigation.

G. A mutation to maltose utilization

During the carbohydrate tests some cases of carbohydrate utilization with a mutational character occurred.

Table 7. Growth of *E. myrtilli* "vit. id." and its maltose-mutant on different carbon sources after 24 and 16 days, respectively.

Tested compound	Dry weight, mg	
	Parent strain (24 days)	Mutant strain (16 days)
No carbon compound	1	2
Glucose	92	114
D-Arabinose	7	7
Xylose	70	50
Cellobiose	76	52
Maltose	3	119
Starch	0	0
Lactic acid	12	9
Citric acid	5	5
Arbutin	14	6

One of these, a mutation to maltose utilization by *E. myrtilli* "vit.id." 4, which appeared after three weeks in one of a series of six flasks of maltose medium, was isolated and studied.

The result of the carbohydrate test presented in Table 7 shows that the mutant and the parent strain had the same power to utilize the tested compounds except maltose. Because of organic impurities in maltose, a small growth of the parent strain also occurred on the maltose media. The two strains were vitamin autotrophic.

Apart from the fact that the mycelia of the mutant strain were somewhat more vigorous, the morphological appearance of the two strains was similar, with similar conidia formation (Plate 1). Besïdes the different capacities to utilize maltose, the mutant had more rapid growth and lacked pigment synthesis.

It is doubtful if the enzyme responsible for the hydrolysis of maltose is specific to this reaction. If the hydrolyzing enzyme has complex functions, it is likely that a single mutation will cause multiple biochemical changes.

The mutant strain has also been studied serologically p. 79.

H. Tilletiopsis, a contaminating fungus

In isolating *Exobasidium*, it was sometimes observed that, in addition to the basidiospores of *Exobasidium*, spores of *Tilletiopsis* occurred in the sporeprint from a diseased plant. As *Tilletiopsis* resembled the *E. vexans* strains in cultural characters and in morphology, e.g., both formed ballistospores on the agar media

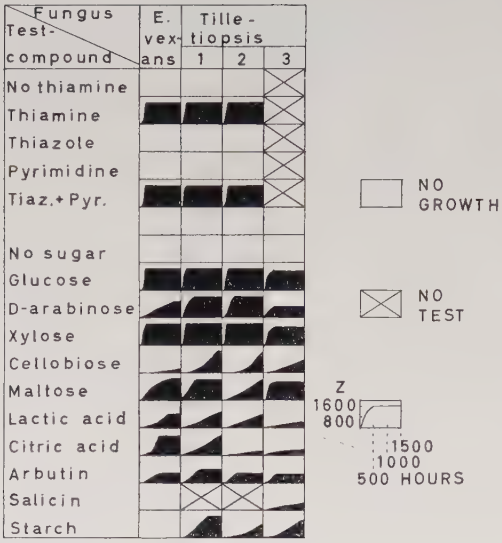


Fig. 18. The ability of *E. vexans* and *Tilletiopsis* spp. to grow on thiamine and its components as the only vitamin, and to utilize different carbon sources. Medium 2 and 3, respectively, were used. The growth curves are based on measurements of turbidity.

(Plate 1), it was of interest to study this contaminating fungus further. In the present investigation, *Tilletiopsis* strains have been isolated from *Vaccinium oxycoccos* both in Sweden and in Germany, and their physiology has been compared with that of *E. vexans*. In spite of the morphological similarities between the two forms their physiological properties differed. Fig. 18 shows that only *Tilletiopsis* spp. utilize starch and salicin. None of five *E. vexans* strains utilized starch.

The studied *E. vexans* strains have been isolated by Dr. Graafland in Baarn from diseased tea plants sent to him by air from Indonesia. Because of his death he did not manage to complete the study of this material, and it was afterwards sent to me. The cultural and morphological similarities found between *E. vexans* and such a common contaminating fungus as *Tilletiopsis* can indicate that confusions may have arisen in isolating the fungi. On the other hand, the observation of Ezuka (1955) that a culture of *E. vexans*, which could infect its host, produces "basidiospores" in culture agrees with observations on my strains of *E. vexans*. The relationship between *E. vexans* and *Tilletiopsis* and the other *Exobasidium* forms has also been studied serologically and is discussed on p. 80.

I. Taxonomic aspects

1. Nutritional characters of the fungus

In the previous part of this report the results were presented without discussing their taxonomic value. The ability of a large number of strains, representing different species and host-races, to assimilate or synthesize about thirty selected

compounds was studied. In particular, those compounds where chosen that Wickerham and Burton (1948) found useful in differentiating yeasts. The biochemical activities that occur in different organisms are greatly diversified, and many enzyme systems—constitutive as well as adaptive—are active in synthesis and assimilation. The result of the nutritional tests on *Exobasidium* showed not only activities that were either present or absent in all strains, but also enzymeactivities that occurred only in some strains. These latter were used for separation of the taxa in *Exobasidium*. As can be seen in Fig. 16 almost all the host-races, that is to say strains that originated from one host and also caused a similar disease picture, as a rule had constant and characteristic physiological properties under the prevailing, standardized conditions. Exceptions will be discussed later.

In spite of the fact that the culture conditions were optimal for the assimilation of glucose, nothing is known about whether the medium was right for utilization of other carbon sources. At least the synthesis of thiamine by *E. vaccinii* "vit.id." and "myrt." was influenced by changes in the medium. Even if new physiological activities appeared after altering the medium or after extending the incubation period, they need not invalidate the results obtained. These were reproducable when the experiment was repeated, and only small variations occurred in the growth within the same strain. It is known that the yield can be influenced by the amount of the inoculum (Meyrath 1963) and by the variation in aeration of the shake cultures. These factors are also difficult to keep constant, and may explain some of the small variations between different strains belonging to the same host-race. It is safest to base the delimitation of the taxa on absolute properties, e.g., growth or not growth, but the appearance of the growth curves also often adds valuable information about a characteristic physiological response. For example, all 18 strains of *E. vaccinii* "vit.id." adapted to xylose utilization after 300–500 hours; the strains of *E. vaccinii* "oxyc." started growth on pyrimidine media after about 500 hours. Thiamine synthesis was more effective by *E. myrtilli* "vit.id." than by *E. myrtilli* "myrt."; the stages at which growth was stopped differed on arbutin media, depending on different tolerances towards the accumulating hydroquinone.

With a few exceptions the tested physiological activities were characteristic within each host-race. The physiological data of taxonomic interest from the different host-races are summarized in Table 8, and from this table it can be seen that there was complete physiological agreement in only 3 cases, between *E. Karstenii* and *E. myrtilli* "myrt.", between *E. vaccinii* "oxyc." and *E. vaccinii* "ulig.", and between *E. vaccinii* "myrt." and *E. vaccinii* "Arct.". The difference between the two first mentioned host-races and *E. myrtilli* "Andr." was small. There were only dissimilarities in the capacity to split arbutin. Each one of all the other host-races had a characteristic physiological response pattern. The resemblance in response among some of the host-races does not necessarily

Table 8. Physiological characters of taxonomic value for the delimitation of taxa in *Exobasidium*.

The strains were tested on a medium without thiamine or with a component of thiamine. Their ability to utilize carbon sources and to split arbutin was also tested.

Strains	No. of strains	No thiamine	Thiazole	Pyrimidine	D-Xylose	Cellobiose	Maltose	Lactic acid	Citric acid	Arbutin	Salicin	Dulcitol	Hydroquinone	Arbutin splitting
E. angustisporum	3	−	−	−	+	−	−	+	+	+	+	(+)	+	+
E. cassiopes	2	+	+	+	−	+	−	+	−	+	+	−	−	+
E. japonicum	4	+	+	+	+	+	+	−	+	−	+	±	−	+
E. Karstenii	6	±	±	+	+	−	−	+	+	+	−	−	−	+
E. myrtilli														
— "Andr."	2	+	+	+	+	−	−	+	+	−	−	−	−	(+)
— "myrt."	5	+	+	+	+	−	−	±	±	+	±	0	0	+
— "ulig."	1	+	+	+	+	−	−	+	+	−	−	0	0	−
— "vit. id."	7	+	+	+	+	+	−	±	±	+	+	0	0	+
E. rhododendri	1	−	−	+	(+)	+	−	−	+	+	+	−	0	0
E. vaccini														
— "Arct."	5	±	±	+	+	+	−	+	−	+	+	−	+	+
— "myrt."	1	−	−	(+)	+	+	−	+	−	+	+	−	0	+
— "oxyc."	4	−	−	+	±	+	−	+	−	−	−	−	−	+
— "ulig."	6	−	−	+	±	+	−	+	−	−	−	−	−	+
— "vit. id."	18	−	−	−	+	−	+	−	+	−	−	−	−	−
E. vexans	5	−	−	−	+	+	+	+	+	+	−	−	0	+
E. uvae ursi	2	+	+	+	+	+	−	−	−	+	−	−	−	+

+ = Growth or splitting capacity.
(+) = Growth or splitting capacity after 1000 or 350 hours, respectively.
± = Growth in some strains and not in others.
− = No growth.
0 = No test.

mean that these are physiologically identical, because it is possible that tests of more activities might separate even these host-races. The hypothesis that the physiology of the pathogen during a single life cycle on the host is influenced by the nutritional status of the host and new enzyme systems induced in the fungus, was contradicted by the following facts: *Andromeda polifolia*, *Arctostaphylos uva-ursi*, *Vaccinium myrtillus*, *Vaccinium uliginosum* and *Vaccinium vitis-idaea* were all infected by at least two different species, e.g., *E. myrtilli* and *E. vaccinii*, but the physiological agreement between the strains from the same host was not greater than between strains from different species of host. It can also be

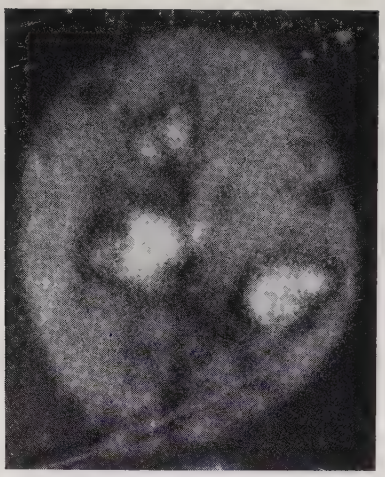


Photo 4. A spore print from a leaf of *Vaccinium vitis-idaea* infected by both the systemic *E. myrtilli* and the gall-producing *E. vaccinii*. The whiter parts in the spore print arise from the more rapid growing spores of *E. vaccinii* "vit. id.".

stated that induced enzyme activities deadapt and adapt depending on the absence or presence of the inducer in the medium.

There are some interesting facts about *E. myrtilli*. This fungus usually occurred on *V. vitis-idaea* or on *V. myrtillus*, especially on plants close to large boulders. In two cases *E. myrtilli* was isolated from the two hosts growing in the shelter of the same boulder, without any other *E. myrtilli* being seen in the vicinity. In spite of the similarities in habitat and disease picture the two host-races differed physiologically from each other. *E. myrtilli* "vit.id." alone utilized cellobiose and produced a pigment, and it had also more effective thiamine synthesis than *E. myrtilli* "myrt.". The dissimilarity between the different host-races was also shown by their different, characteristic maximum temperatures.

The most probable explanation of these results is that the different hosts are attacked by different physiological races, and that there is a genetic basis for the physiological specialization.

2. DISEASE SYMPTOMS OF THE HOST

At least in *Vaccinium vitis-idaea*, which is attacked by different species (causing hypertrophic spots or systemic attacks with inconsiderable hypertrophy), the different disease pictures seem to be the result of the different physiological characteristics of the infecting strains, e.g., production of different auxins. In very rare cases, when the gall-producing species, e.g., *E. vaccinii* "vit.id.", attack very young buds or shoot primordia seriously, systemic attacks may result, but these differ clearly from the normally systemic species by the greater hypertrophy.

Further there were two cases found in which the same host plant was attacked by 2 different species, *E. vaccinii* "vit.id." 17 and 18 and *E. myrtilli* "vit.id." 6 and

7. In both cases *E. vaccinii* produced a distinct hypertrophic spot on the leaf already infected by the systemic form. The induction in the same leaf of the different disease symptoms, characteristic for the two fungi, reflected the differences in the morphogenetically active compounds. Photo 4 shows a spore-print from the doubly infected leaf. The whiter spots, composed of the more rapid growing spores of *E. vaccinii*, mark the distribution of the galls. The spore morphology was also characteristic of the two species. Tests of single spore as well as of multispore isolations of the two species *E. vaccinii* "vit.id." 17 and 18, and *E. myrtilli* "vit.id." 6 and 7, showed that the two forms retained their physiological properties. There was no sign that any recombinants of the two species occurred when they parasitized the same leaf.

There are also differences in the disease symptoms between the two species occurring on *Arctostaphylos uva-ursi*, but the dissimilarities between these as regards hypertrophy were not as distinct.

V. MORPHOLOGICAL AND CYTOLOGICAL STUDIES

A. The size of the basidiospores

The morphology of the basidiospore is an important character on which the taxonomy of *Exobasidium* is based. As there are many host-races represented in this investigation, and as some of them have been grouped together within the described type species without any information about the exact shape and size of the basidiospores, it was of interest to compare further the basidiospores of the different host-races. It was also valuable to get an idea about the stability of this character.

Savile (1959) has studied most of the forms represented in this investigation, but as most of them had basidiospores of about the same size they were presented without discrimination between them. Further, it is often difficult to compare information given by different authors.

Plants infected with *Exobasidium* were collected from the north, middle and south of Sweden. The diseased parts were placed at 20°C in petri dishes, where *Exobasidium* disseminated the basidiospores on a malt agar medium. The spores with the shortest latent period of germination germinated after 3 hours. After 3 hours the spore prints of different origins were photographed, using a Zeiss-"Photomikroskop Mikromat" at 405 x. The basidiospores were measured after further enlargement by projecting the negative film and the average values of their width and length are presented in Fig. 19. Spores from infections of different appearance were studied. The average value was based on 15–40 spores per spore print, and each plant and locality was represented by only one value. All host-races are not represented as it has been difficult to obtain viable spores from rare or distant forms.

No comparison has been made between the size of the viable spores in a humid environment and that of the spores from dried herbarium specimens. There are reports (Hesse 1876 and Brodie 1936) which show that germination is accompanied by swelling of the basidiospores. Microscopic inspection of the *Exobasidium* spores did not reveal any visible changes during the first few hours on malt agar. This indicated that the observed differences between the host-races and between the species are characteristic properties and cannot be modifications which arise during germination.

Fig. 19 shows that in two species, *E. vaccinii-uliginosi* and *E. uvae ursi*, the size of the basidiospores is clearly different from that of the other species. Cha-

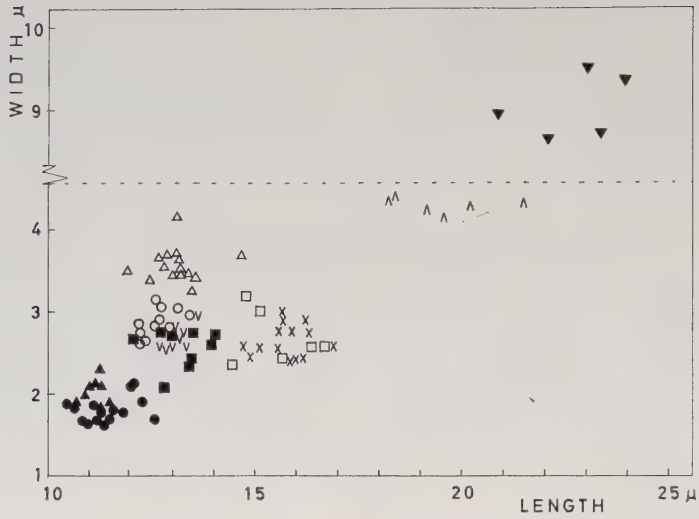


Fig. 19. Size of basidiospores from different *Exobasidium* host-races obtained from collections of infected leaves. Mean values of the length and width of spores discharged onto an agar medium are plotted.

□ = E. Karstenii
▲ = E. myrtilli "Andr."
■ = E. myrtilli "myrt."
● = E. myrtilli "vit. id."
V = E. vaccinii "Arct."
○ = E. vaccinii "oxyc."
△ = E. vaccinii 'ulig."
× = E. vaccinii "vit. id."
Λ = E. uvae ursi
▼ = E. vaccinii-uliginosi Boud.; from Vaccinium vitis-idaea

racterization of the other species by the size of the basidiospores is more doubtful. A great variation in size and shape can be seen within the species, but within each host-race the variation is slight. Thus each host-race has basidiospores of similar appearance. *E. myrtilli* has the smallest basidiospores. The size of the spores varied among its host-races, and the spores of the host-race with the largest spores were of similar size to spores of the host-races of *E. vaccinii* with the smallest spores. Thus it is difficult to distinguish between the spores of *E. myrtilli* "myrt." and those from *E. vaccinii* "Arct." and *E. vaccinii* "oxyc.". Similarly it is impossible to separate the basidiospores of *E. vaccinii* "vit.id." from those of *E. Karstenii*.

The basidiospores of *E. vaccinii* "ulig." and *E. vaccinii-uliginosi* are the broadest in relation to their length.

B. Germination and mode of growth

Woronin, in 1867, was the first to notice that there was both conidial and mycelial growth in *Exobasidium vaccinii*. The basidiospores that germinated in water

or a saturated atmosphere outside the host formed short germ-tubes from which conidia were produced, and these continued to grow by budding. On the other hand, the basidiospores that germinated on the young leaves of the host did not produce conidia or secondary spores but developed long germ-tubes which penetrated the host, either through the stomata or by piercing the epidermis. Inside the host the fungus continued to grow in a mycelial form.

This reversible change between a mycelial and a unicellular growth type is called dimorphism. Later work on *E. vaccinii* (Brefeld 1889, Graafland 1953) confirms Woronin's observation, but there are also reports of an *Exobasidium* species that has no dimorphism. Gaad and Loos (1948, and 1950) and Graafland (1953) found that basidiospores of *E. vexans* germinated with long germ-tubes without any production of conidia. In a later work Ezuka (1955) stated that this species too could multiply by budding. The occurrence of different germination types of basidiospores justified a further study of the frequency and the stability of the two types.

Germination was studied on an agar substrate containing malt extract or medium 2. pH was 6.0 and 5.0, respectively. The diseased plants discharged their basidiospores on the agar, where germination occurred. There were strains with or without dimorphism in my material; this could be seen after 1–2 days on the agar medium.

The following host-races had basidiospores that grew into mycelia (Plate 3): *E. cassiopes*, a form with amphigenous hymenium, all host-races of *E. myrtilli*, and *E. vaccinii-uliginosi* Boud. see later.

The following host-races formed only conidia (Plate 4): *E. angustisporum*, *E. Karstenii*, all host-races of *E. vaccinii*, *E. uvae ursi*, and *E. cassiopes*, a form with hypophyllous hymenium see later.

The mode of germination was stable and characteristic of each host-race on the agar media used and this behaviour seems to be a valuable taxonomic character.

In *Exobasidium* not only the germination was characteristic, but also the continued growth on agar media, where the conidial or mycelial growth form was maintained. In *E. japonicum* and *E. rhododendri*, two species in which the germination has not been studied, the growth on agar was mycelial and conidial, respectively. From the mycelium of the *Exobasidium* host-races with a mycelial growth, there was conidia formation of a characteristic appearance on agar media (Plate 1). *E. vexans* was different in this respect, in that ballistospores were formed, which gave rise to short mycelia producing more ballistospores.

The mycelial growth was influenced by the environment. In Photo 5, neighbouring colonies, mycelial as well as conidial, induced the mycelial form to conidial growth. Furthermore, the conidial production was stimulated by cultivation in liquid medium. *E. cassiopes* and *E. myrtilli* "Andr.", which grew as mycelia

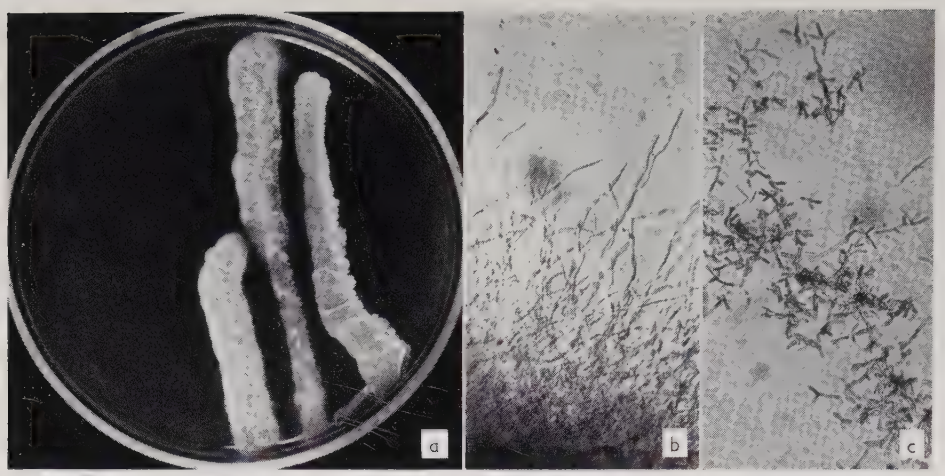


Photo 5. Influence of adjacent colonies on the mode of growth. (a) To the left, two colonies of *E. cassiopes*, to the right a colony of *E. vaccinii*. The margin of the colonies of *E. cassiopes* (b) without and (c) with an adjacent colony.

on agar, changed readily to conidial growth when they were transferred into liquid medium. Even *E. myrtilli* "myrt." changed after several transfers, while *E. myrtilli* "vit.id." was most stable and grew as a mycelium with only a sparse conidia production.

Germination and growth of *Exobasidium* is of the same type as that of *Gloeotinia temulenta*. Griffith (1958) showed that the mycelial or conidial state in this species was inherited but that the mode of growth was affected by the environment.

The conidial forms were much less influenced by the environment than were the mycelial ones, but nevertheless there were discernible morphological differences within the same strain growing on different media.

Colour and texture was characteristic of each host-race. As can be seen from Plate 2, the greatest variation occurred in *E. angustisporum*; otherwise the variation was insignificant. The differences in the size of the colonies depend on varying amounts of inoculum. The surface of the colonies of *E. vaccinii* "oxyc." and *E. vaccinii* "ulig." was first smooth and glossy but became wrinkled in old cultures. Plate 2 illustrates, the variation between the host-races and the uniformity in morphology within a host-race. This was independent of the composition of the medium.

The germination and growth of basidiospores of *E. cassiopes* with hypophyllous hymenium and of *E. vaccinii-uliginosi* differs from that of the other *Exobasidium* strains studied. The former germinated normally with a small germ-tube, which produced a small number of conidia, but these did not continue to grow. Not

even the media on which the amphigenous form thrive could stimulate them to grow.

The basidiospores of *E. vaccinii-uliginosi* did not germinate until after 4–6 months (Plate 3). The spores that germinated had been kept on water agar at 5°C. Experiments at 10 and 20°C with water agar, with aqueous extracts of the host, with chelates and with different nutrient media were done, but during the two to three weeks the basidiospores were studied no germination was observed. On most media the contents of the spores became granulated, the ends of the spores formed bulges, and many of them burst. Although the nutritional environment was varied, this fungus was another that could not be cultivated. Growth ceased after the development of long germ-tubes. This stage can be seen in Plate 3.

C. Cytology

Eftimiu and Kharbush (1927) studied thoroughly the cytology of *Exobasidium japonicum*, *E. myrtilli*, *E. vaccinii-uliginosi*, *E. rhododendri*, and *E. uvae ursi*. They found that the intercellular mycelia were dicaryotic, as well as the young basidia. The nuclei of the basidium fused into a diploid nucleus, and then there were usually two divisions. The resulting four haploid nuclei each passed into a basidiospore via the sterigma. The basidiospores were uninucleate, but sometimes variable in number.

Later, Graafland (1960) published an investigation about *E. japonicum*. He showed in his work, which completed the investigations of Eftimiu and Kharbush, that both the basidiospores and the conidia of this species were uninucleate. He also succeeded in infecting the host with a culture that originated from a single uninucleate conidium, thus indicating that *E. japonicum* was homothallic.

Investigations have shown that a single-cell culture originating from a conidium with two genetically different nuclei is more variable than a culture from a uninucleate conidium (Hansen 1938). To determine the number of nuclei per cell in the *Exobasidium* strains the following study was made, mainly of the conidia and the basidiospores.

The nuclei of conidia that had been produced in liquid medium 3 and of germinating basidiospores were stained with azure A after fixation for 25 min in alcohol-glacial-acetic acid and hydrolysis for 5–10 min in 1 N HCl at 60°C (v. Hofsten 1959). The material was fastened to the slides by slight drying.

The conidial forms could easily be stained and studied by this method, whereas the stained nuclei of the mycelia were too diffuse, and the mycelia too fine for the septation to be seen. *E. angustisporum*, *E. cassiopes*, *E. Karstenii*, and all the host-races of *E. vaccinii* were studied, and all of them had uninucleate conidia. In Plate 5 the conidia can be seen with one stained nucleus per cell, except in the budding cells where there are two nuclei. These results indicate that, at

least in these forms, each single-cell culture must be genetically uniform as it originates from a single uninucleate conidium, and further that the dicaryotic state does not occur outside the host.

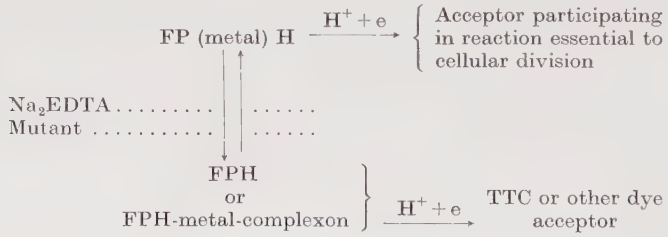
When the spores had been discharged from the galls and germinated on the malt-agar medium, no signs of fusion were seen, either between spores from different collections of one host-race or between spores from different host-races.

D. Dimorphism

1. Introduction

Many studies have been devoted to the problem of dimorphism, and many investigators have been able to change the mode of growth of an organism by varying its environment. However, the problem has not often been treated biochemically. Nickerson and co-workers, for example, have traced a connection between biochemistry and morphogenesis. My study of dimorphism in *Exobasidium* was based to some extent on their results, summarized below.

Nickerson (1954) compared a filamentous mutant of a pathogenic yeast—*Candida albicans*—with its parent strain and he found that during growth the mycelial form reduced triphenyltetrazoliumchloride (TTC) while the parent strain did not. After an addition of EDTA to the parent form this strain also reduced the compound and became filamentous. He proposed that EDTA blocked a flavoprotein enzyme which was active in cell division. Whether this block occurred through a mutation or by action of a chelate, the fungus changed to mycelial growth, and TTC was reduced and coloured. In the scheme below (from Nickerson 1954) the proposed block of a flavoprotein enzyme is shown.



In spite of the waste of the reductive capacity, there was no decrease in the growth rate or in the respiration of the mutant form. Thus the morphogenetic phase can be separated from the growth phase.

Later Nickerson and Falcone (1956) demonstrated the presence of a very active "division enzyme" in the parent strain and they showed also that similar enzyme preparations from the non-dividing strain gave only a feeble activity. The enzyme, a protein disulphide reductase, reduced the disulphide linkages of the cell-

wall protein and during this process the cell-wall was locally softened and a bud could arise. Analyses of the cell-wall proteins showed that they were of a pseudo-keratin type with more than 2 per cent sulphur. Preliminary evidence indicated that the enzyme was a flavoprotein. It is not known whether there are other species with biochemical behaviour similar to *Candida albicans* in the morphogenesis, but it is known that –SH compounds dominate in forms with a conidial or yeast-like mode of growth (Scherr and Weaver 1953).

The investigation of the dimorphism in *Exobasidium* had the following principal aims:

1. To look for morphogenetically active compounds in the host.
2. To test known morphogenetically active compounds.
3. To find out the influence of cultural variables.
4. To study the reducing capacity of the culture.

2. MORPHOGENETICALLY ACTIVE COMPOUNDS IN THE HOST

In the study of active compounds in the host which could transform conidial into mycelial growth, special interest was devoted to phenol-phenol derivatives and auxins. Phenols and corresponding quinones function as redox system (Hewitt 1950) and can theoretically interfere with intracellular reductions, e.g., those that lead to –SH groups in the cell wall protein. It had also been mentioned earlier that arbutin is stored in ericaceous plants, and that this compound can be utilized both by the plants and the fungi. In these instances the hydroquinone was released. Tyrosinase and laccase, both of which oxidize phenols, are found both in higher plants and microorganisms. Wosilait and Nason (1954) found that p-bensoquinone oxidized cysteine and glutathione spontaneously.

It has also been shown that auxins in some cases caused an increased tendency to hyphal growth (Gruen 1959).

In spite of the fact that the conidial growth of *Exobasidium* was transformed into filamentous growth when the fungus started a parasitic life, it was impossible to demonstrate compounds in the young host that induced filamentous growth. Neither extracts sterilized by filtration, nor the intact shoots imbedded in the agar had any effect on the morphology of the conidia. To avoid substances inhibiting any possible active compounds and to be less dependent on the concentration of the compound, a concentrated extract was chromatographed.

In separating phenols and auxins from the extracts, the directions given by Reio (1958) and Nitsch (1956) were followed. The paper strip with the separated extract was ether-sterilized and applied to a large petri dish with agar and conidia. Growth of the conidia close to the strip was studied and the influence of the diffusing compounds was followed microscopically through the bottom of the dish.

In these experiments no morphogenetically active compounds, which could affect the conidial growth of *E. vaccinii* "vit.id.", were detected in *V. vitis-idaea.*

Neither was the mode of growth changed by cultivation on medium 2 with quinones added. On the other hand, small amounts of p-bensoquinone, 1:4 and 1:2-naphtaquinone at 1–10 mg per l inhibited growth. These compounds did not affect the morphology, but the results need not exclude the possibility of quinone systems with other redox potentials being effective.

Exobasidium was more tolerant towards hydroquinone than towards quinone and the inhibiting concentrations were between 100 and 1000 mg per l. The influence of hydroquinone on morphogenesis was insignificant.

3. THE EFFECT OF KNOWN MORPHOGENETICALLY ACTIVE COMPOUNDS

Experiments with chelators. In many enzymes, alkali metals and ions of heavy metals function as coenzymes, and as has been mentioned earlier, Nickerson transformed *Candida* into a filamentous growth by chelating a metal ion essential for enzyme activity.

In my experiments ethylenediaminetetraacetic acid (EDTA) was used. It was autoclaved separately and afterwards added to medium 3, pH 5.3. The two extreme growth forms, *E. vaccinii* "vit.id." and *E. myrtilli* "vit.id." were cultivated in shake culture.

The growth of both fungi was inhibited by the chelator to about the same degree, but only the filamentous form was affected morphologically. As can be seen from Photo 6, $5 \cdot 10^{-4}$ M EDTA transformed *E. myrtilli* "vit.id." into a conidial state and blocked the pigment synthesis. pH and composition of the test media were different from those of the media used by Nickerson in his studies of *Candida.* Therefore, it is difficult to compare the chelating effects on *Exobasidium* with those on *Candida.*

A similar effect has been reported by Lougheed (1963). He found that the mycelial growth of *Hirsutella gigantea* could be transformed into a yeast-like growth by EDTA, and he also reversed this chelating effect by the addition of metal ions; Mn^{++}, Fe^{++} or Ca^{++}.

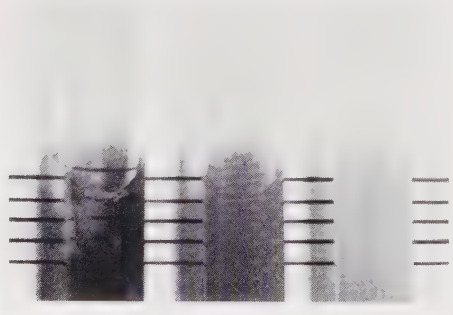


Photo 6. The effect of EDTA on growth and pigment production of *E. myrtilli* "vit. id.". From the left, the tubes with medium 3 contain the following additions: 0; 10^{-4}; and $5 \cdot 10^{-4}$ M EDTA.

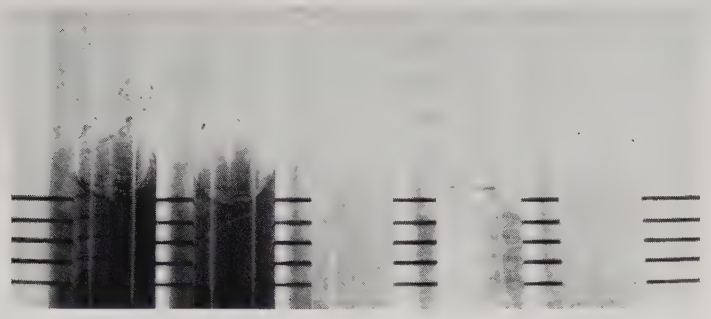


Photo 7. The effect of cysteine and glutathione on growth and pigment production of *E. myrtilli* "vit. id.". From the left, the tubes with medium 2 contain the following additions: 0, 10^{-3} and 10^{-2} M glutathione; 10^{-3} and 10^{-2} M cysteine.

Experiments with sulfhydryl compounds. There are many examples of sulfhydryl compounds favouring unicellular growth. Cochrane (1958 p. 10) lists some species to which an addition of cysteine induced non-mycelial growth in mycelial forms. In *Ophiostoma*, on the other hand, cysteine did not increase conidial growth (v. Hofsten and v. Hofsten 1958).

To investigate the effect of sulfhydryl compounds on *Exobasidium*, mycelial or conidial forms were cultivated in shake culture with glutathione and cysteine added. The two compounds were sterilized by filtering and medium 2 was used. The final concentrations were 10^{-2} M and 10^{-3} M.

In the filamentous forms of *Exobasidium* both cysteine and glutathione favoured non-mycelial growth, while the morphology of the conidial forms was unaffected. As can be seen in Photo 7, 10^{-2} M cysteine and gluthathione transformed the most extreme mycelial growth of *E. myrtilli* "vit.id." into a pure conidial state and at the same time stopped the pigment production. Glutathione was somewhat less effective. Cysteine as well as glutathione inhibited both growth forms at the concentrations used, but other workers have shown that the promotion of the conidial state is not always followed by inhibition of growth.

Nickerson and Chung (1954) demonstrated that colonies of *Candida albicans* induced yeast-like cells in the adjacent colony of the mycelial mutant. They ascribed this behaviour to the ability of the yeast to exude sulfhydryl compounds, probably cysteine. The earlier described tendency of conidial or mycelial forms to give rise to conidial growth in colonies of adjacent filamentous *Exobasidium* strains may be caused by increased concentrations of exuded sulfhydryl compounds, but this has not been investigated.

4. THE INFLUENCE OF CULTURAL VARIABLES

Supraoptimal temperatures stimulated filamentous growth, while low temperatures favoured a conidial state. This thermal dimorphism was most pronounced

in *E. Karstenii* and *E. cassiopes*. A changed relation between oxygen and carbon dioxide content in the environment of the fungus did not change the growth form of *E. vaccinii* (10 % CO_2 + 90 % air or 50 % O_2 + 50 % air). pH of the medium did not affect the conidial growth. However, the carbon source had a large effect. The compounds that gave good growth also stimulated the maintenance of the conidial state, while the poor carbon sources often induced mycelial growth. Mannitol strongly favoured conidial growth in liquid cultures of *E. cassiopes*.

5. THE REDUCING CAPACITY

As was mentioned earlier (p. 60) there was a difference between the dyereduction by *Candida albicans* and that by its mutant. The latter lacked an effective flavoprotein enzyme which was active in cell division by reducing sulphydryl compounds in the cell-wall protein. This deficiency caused a waste of the metabolically generated reducing capacity, which could reduce dyes with redox potentials more positive than the flavoprotein enzyme.

As in Nickerson's experiment (Nickerson 1954) redox indicator dyes were used when the reducing capacity of the two growth forms of *Exobasidium* was studied. Nickerson's GGY medium and my medium 2 were used. pH was adjusted to 6.0. The dyes listed below were sterilized by filtration.

Thionine	E'0 = + 0.063 v
Methylene blue	E'0 = + 0.011 v
TTC	E'0 = − 0.083 v
Nile blue A	E'0 = − 0.125 v
Neutral red	E'0 = − 0.325 v

Only thionine was reduced. No differences were observed between the conidial and the filamentous forms of *Exobasidium* in the capacity to reduce dyes.

Thus it seems that the morphogenesis of *Exobasidium* is regulated by a different or modified mechanism than that of *Candida*.

E. Taxonomic aspects

Compared with the less complicated biochemical activities, which are usually either present or absent, the major morphological characters of an organism are more difficult to express exactly. They are the result of many interacting processes which are determined by a complex of genetic factors and influenced by the environment. There are also many workers who consider that the morphological characteristics are more variable than the physiological ones (e.g., Rettger 1939, Skinner 1947, and Griffith 1958). However, there are also examples of workers of the opposite opinion (e.g., Snyder and Hansen 1954). In these cases such complicated and easily modified physiological activities as pigmentation and rate of growth or pathogenesis were studied.

In the present investigation the reliability of both physiological and morphological characters has been assessed.

The variation in the size and shape of the basidiospores of *Exobasidium* within each host-race was small. The differences between the host-races were often not significant, but in many cases it was possible to separate the different host-races on the basis of basidiospore size (Fig. 19).

Another morphological character is the mode of germination of the basidiospores. Griffiths (1958) showed that the mycelial and conidial form of *Gloeotinia temulenta* was inherited but that the mode of growth was affected by the substrate. This is also the case in *Exobasidium*, where some of the species have mycelial growth, and some conidial. In addition, at least in *E. vaccinii*, there were other small and constant characters in mode of germination, typical for the different host-races (Plate 4).

As is partly evident from Plate 2, the cultural characters (colour, colony margination, texture and mode of growth) were constant within most host-races, and on the basis of the differences in colony appearance it is possible to classify the strains into host-races, except for strains of *E. vaccinii* "myrt.", *E. vaccinii* "oxyc.", *E. vaccinii* "ulig." and *E. uvae ursi*. All these host-races had similar colonies, but *E. uvae ursi* had larger conidia and could thus be distinguished.

It has been shown (ch. IV.I) that the isolated *Exobasidium* strains can be classed together into groups with similar physiological activities, and these groups correspond with the host-races. A delimitation of taxa based on morphological criteria gives similar results.

This is not surprising, because both morphological and physiological characters are based on the genetic make-up. The problem is to assess the variation in the genus or species studied, and to find the characters which are stable and characteristic of the taxon. As the environment easily modifies morphological as well as physiological characters, it is important to diminish its influence, by carrying out the comparative studies under controlled conditions.

The physiological data have been obtained under constant and controlled conditions, while the development of some important morphological characters has been studied in less well defined conditions. As the basidiospores measured in the present investigation were formed by fungi parasitizing different hosts, often representing different genera, it is possible that their different nutrient status and metabolism may also have affected the size of the basidiospores.

Pound and Williams (1963), showed that *Albugo candida* formed spores of different size, when the same fungus parasitized different species. In *Sclerotinia trifoliorum*, the length of the ascospores was modified by the substrate (Björling 1951). As the influence of host genotype on the basidiospores has not been studied, it is difficult to say whether the small dissimilarities in basidiospore size between the host-races depend on differences in the host or in the fungus. The

Exobasidium forms attacking the same species, and nevertheless always producing basidiospores of different appearance, must be regarded as different forms, e.g., *E. Karstenii—E. myrtilli* "Andr.", *E. vaccinii* "vit.id."—*E. myrtilli* "vit.id." —*E. vaccinii-uliginosi*, and *E. vaccinii* "Arct."—*E. uvae ursi*.

Physiological specialization of a parasite need not always be followed by apparent morphological changes, e.g., *Puccinia* (Fletcher 1963). In *Exobasidium* there is both a physiological and a morphological specialization. Some of the host-races in *E. vaccinii*, e.g., *E. vaccinii* "vit.id." have physiological and morphological properties which are distinctly different from those of the other host-races. On the other hand, *E. vaccinii* "oxyc." and *E. vaccinii* "ulig." agreed in physiology and cultural characters, but differed in the size of the basidiospores, and somewhat in the configuration of the sprouting conidia at germination.

VI. INOCULATION EXPERIMENTS

A. Introduction

Since Woronin (1867) reported that *Vaccinium vitis-idaea* could be infected by basidiospores of *Exobasidium*, more information about the pathogenic behaviour of this genus has been obtained.

Lockhart (1958) studied a species causing systemic infections in *Vaccinium myrtilloides* and *V. angustisporum*. The symptoms of the disease did not appear at the place of the inoculation, but the pathogen grew inside the plant, penetrated the stem and reached the rhizome. Only after 3–7 months did the disease appear, at first in the shoots developing from the stem and later in the shoots from the rhizome. Earlier, Hilborn and Hyland (1956) had stated that the fungus penetrated the cuticle and spread in the cortex of the host. The mycelium could live for several years in the rhizome and the disease appeared when new shoots were formed from the rhizome.

Göttgens (1959) and Graafland (1960) showed that in *E. japonicum* both the basidiospores and the conidia in culture could infect the host. The conidia were still virulent after 6–7 years in culture. Only very young leaves, the lamina of which was not fully expanded, were infected. By inoculating still dormant buds, Graafland managed to keep the fungus alive on the host for six months. The incubation time depended on the development of the bud. When the bud began to burst, growth of the fungus also started. The life cycle of the gall-causing *Exobasidium* species is about 3–4 weeks (Woronin 1867, Graafland 1960).

Inoculation experiments (Graafland 1960) have shown that *E. japonicum* is not narrowly specialized, as the fungus could also attack other Azalea cultivars than the one from which it was isolated. On the other hand, cross inoculations on Azalea cultivars and *Vaccinium vitis-idaea* with spores of *E. japonicum* and *E. vaccinii* were not successful, in spite of the similar disease symptoms caused by the two pathogens. The fungi could only infect their usual host. Richards (1896) showed that the same *Exobasidium* species infected both *Gaylussacia resina* and *Andromeda ligustrina*. Graafland's investigation (1960) indicated that a high degree of specialization of pathogenicity can occur in *Exobasidium*, whereas Richards (1896) found less specialization.

The morphological differences between the *Exobasidium* species studied in the present investigation are small, and, as was mentioned earlier, most of them could, according to Savile, be brought together into *E. vaccinii* (Savile 1959).

However, within the species studied there are also morphologically characteri-

stic sub-units, representing the host-races. These host-races could be nutritionally characterized, but it remained to investigate if they were specialized in their pathogenicity.

Hence inoculation experiments were carried out, partly in the field and partly in the laboratory, where unsterile plants brought in from the field, sterile seedlings germinated in the laboratory, and tissue cultures were inoculated.

B. Field experiments

Early in the spring of 1960, at the time when the different hosts had just started to unfold their buds, the inoculations were made. In the first experiment, *Vaccinium vitis-idaea* was inoculated with different *Exobasidium* species. A conidial suspension of a monospore culture or of a mixture of monospore cultures was injected into each one of 40 buds. The inoculated plants were situated within marked squares. These had about the same number of *V. vitis-idaea* plants and there was at least 20 m between the squares. After five weeks the squares were

Table 9. Number of attacked shoots resulting after 5 weeks of incubation from inoculations of *V. vitis-idaea* plants with monospore or mixed monospore cultures of different *Exobasidium* strains.

40 buds were inoculated.

Conidial suspensions	No. of attacked shoots	
	Locality 1	Locality 2
Control	4	1
	2	1
	8	4
	2	2
E. angustisporum 1	2	0
E. cassiopes 1	—[a]	1
E. Karstenii 1,2,3	—	1
E. myrtilli		
— "vit. id." 1,2,3	—	3
— "myrt." 1,2	—	3
E. vaccinii		
— "vit. id." 1	12	30
— "vit. id." 2	28	31
— "vit. id." 3	30	19
— "vit. id." 4	17	—
— "vit. id." 5	35	—
— "vit. id." 1–5	33	—

[a] No inoculation was made.

Table 10. The total number of galls, 23, 27, 40 and 60 days after the inoculation of 4 hosts with different host-races of *Exobasidium*.

Conidial suspensions	*Vaccinium vitis-idaea*	*Vaccinium oxycoccos*	*Vaccinium uliginosum*	*Arctostaphylos uva-ursi*
E. vaccinii "oxyc." 1–4				
23 (days)	3	0	0	1
27	5	3	0	1
40	8	4	1	1
60	13	8	1	1
E. vaccinii "Arct." 1 + 3				
23 (days)	2	0	0	1
27	3	0	0	1
40	5	0	0	1
60	5	0	0	1
E. vaccinii "vit. id." 4 + 7				
23 (days)	16	0	0	0
27	55	0	0	0
40	96	0	0	0
60	115	0	0	0
Control				
23 (days)	2	0	0	0
27	2	0	0	0
40	3	0	0	0
60	8	0	0	0

inspected and all attacks within the inoculated squares and within the control squares with no inoculations were noted (Table 9).

As the early summer of 1960 was rainy, the *Exobasidium* attacks were numerous. Each control square had one or more galls of *E. vaccinii* "vit.id." type.

Table 9 shows that both monospore cultures and the mixed monospore cultures of *E. vaccinii* "vit.id." constantly caused a large number of attacks, many of which were serious. It is clear that *E. vaccinii* "vit.id." is pathogenic to *V. vitis-idaea* and like *E. japonicum* (Graafland 1960) it is also a homothallic fungus, as monospore cultures produced a typical hymenium with basidiospores on the host. The experiment also indicated that the other *Exobasidium* species could not easily attack *V. vitis-idaea*. Inspections 2–3 or 14 months after the inoculation did not reveal any infections other than of the *E. vaccinii* "vit. id." type. The failure of *E. myrtilli* "vit. id." to infect *Vaccinium vitis-idaea*, which is its usual host, may be connected with the fact that in the low-land *E. myrtilli* "vit. id." has never been observed on plants in the open field but only on weakly growing plants in the shelter of large boulders.

In a locality near Uppsala, the rare hybrid between *V. vitis-idaea* and *V. myrtillus* was inoculated with *E. vaccinii* "vit.id.". In spite of its pathogenicity towards *V. vitis-idaea*, the fungus was not pathogenic towards the hybrid.

In the spring of 1961 another inoculation experiment was done. Squares with *Vaccinium vitis-idaea*, *Vaccinium oxycoccos*, *Vaccinium uliginosum*, and *Arctostaphylos uva-ursi* were sprayed four times every third day with conidial suspensions of *E. vaccinii* "vit.id.", *E. vaccinii* "oxyc.", and *E. vaccinii* "Arct.".

In this year too, spontaneous attacks, both within and outside the control squares were frequent. The only reliable result of this experiment was that *E. vaccinii* "vit.id." was pathogenic on *Vaccinium vitis-idaea* and not on the other species (Table 10). It also seemed as if the other *E. vaccinii* forms were not able to infect *V. vitis-idaea*. The difficulty of mixed cultures of several strains of *E. vaccinii* "oxyc." and of *E. vaccinii* "Arct." to infect their host-plants in these experiments was notable. At the inspection after 27 days the diseased plants were collected and their basidiospores studied. As was expected the appearances of the basidiospores from *V. vitis-idaea*, *V. oxycoccos* and *A. uva-ursi* agreed with those of *E. vaccinii* "vit.id.", *E. vaccinii* "oxyc.", and *E. vaccinii* "Arct.", respectively.

Repeated inoculation experiments with collected plants kept under controlled conditions in a greenhouse have given similar results. *E. vaccinii* "vit.id." was strongly virulent towards *V. vitis-idaea*, but could not infect *V. myrtillus*, *V. uliginosum*, and *V. oxycoccos*. The *Exobasidium vaccinii* host-races isolated from the last three host-plants could not infect *V. vitis-idaea*, but showed at the same time very low virulence towards the species from which they had been isolated, too low to exclude entirely the possibility of the existence of dormant infections.

Earlier experiments have shown that *Exobasidium* can be kept alive for a long time in the rhizome as well as in the dormant buds (Lockhart 1958, Graafland 1960). To avoid the risk of a latent fungus appearing in inoculation experiments, cultivation of the host under sterile conditions was tried. It was also desirable to find a cultivation method that favoured the infection of the plant and was convenient for cross inoculation on a large scale. Experimental inoculations of sterile seedlings and tissue cultures were made.

C. Inoculation of sterile seedlings

In sterile cultivation of the ericaceous seedlings, no mycorrhizas develop and growth is poor (Freisleben 1934). Sterile seedlings of *Vaccinium vitis-idaea*, *V. oxycoccos*, *V. myrtillus*, and *Cassiope tetragona* were cultivated in culture tubes containing malt agar (3 % malt extract, 1.2 % agar and pH 6.0). Twice, with an interval of one week, the young seedlings were moistened with suspensions of conidia. Each host was inoculated with the *Exobasidium* form earlier isolated

from the same species. The tested seedlings, the final length of which was not more than 2–4 cm, were very resistant towards the disease, and it is possible that plants in this phase of development were not suitable for infection. Only one seedling of *V. vitis-idaea* out of the 21 inoculated was hypertrophied. After one month's incubation this plant bore young basidia. Microscopic inspection of the seedlings after 1 or 2 months did not reveal further infections either on *V. vitis-idaea* or on the other species.

D. Inoculation of tissue cultures

It is now possible to produce tissue cultures from most dicotyledons. This cultivation technique has already been successfully used in studies of the relation between parasite and host. Viruses as well as bacteria and fungi have been cultured on tissue cultures. With this technique it has also been possible to cultivate and study obligate parasites (Riker and Hildebrandt 1958). For example, Constabel (1957), and Cutter (1959) showed that *Gymnosporangium juniperi-virginianae* grew both in the aecial and in the telial state on callus.

I intended to inoculate the callus of the ericaceous hosts with different *Exobasidium* strains. If the host callus could support parasitic growth of *Exobasidium*, and a hymenium developed, it would be possible to obtain information about the pathogenic specialization of the different host-races.

There is only one example of induction of callus of *Vaccinium vitis-idaea* so far reported (Hotson 1953), but this callus had a poor growth and survived only one transfer.

In my experiments, pieces of the cotyledons or the epicotyls were taken from young, sterile seedlings of *Vaccinium vitis-idaea* and *V. myrtillus*, and placed on the callus-inducing medium. A Heller medium (Heller 1953), as modified by T. Eriksson, was the best callus-inducing medium. In the Heller medium IAA was replaced by 2:4–D (6 mg/l) and casamino acids (500 mg/l) and coconut milk (100 ml/l) were added.

The induction of callus growth in the *Vaccinium* species took about 3–4 weeks and growth was very slow at first. After some time on the medium, the callus grew more rapidly. This adapted tissue culture was transferred to the agar-medium defined by Murashige and Skoog (1962), which supported good growth. The cultures were at 25°C in the light. The callus of *V. myrtillus* was green and that of *V. vitis-idaea* was pale.

There were at least two ways of controlling the growth of the fungi on the callus. Since it was possible to cultivate the tissue cultures on the defined medium without thiamine, the *Exobasidium* strains requiring thiamine became dependent on the callus for this vitamin. It was also possible to regulate the growth rates of the fungus and the callus relative to one another. At low temperatures the callus

was overgrown by the fungus and killed, but near the maximum temperature of the fungus there was a balance in their growth.

Tissue cultures of *V. myrtillus* and *V. vitis-idaea* were inoculated with the corresponding host-races of *E. vaccinii* and *E. myrtilli*. The tissue cultures were allowed to overgrow the fungi at a high temperature, and after two weeks they were removed to a lower temperature, where the fungus grew out of the callus. The tissue cultures were examined microscopically after 3, 4, 5, 6, 7, and 8 weeks, but no disease symptoms or basidiospores could be seen. The fungi grew only saprophytically. The *E. vaccinii* forms grew in a conidial form and *E. myrtilli* grew in a mycelial form. The metabolism of the callus was probably too changed to be a suitable environment for parasitic growth of *Exobasidium*.

E. Crossing experiments

Eftimiu and Kharbush (1927) stated that the intercellularly growing mycelium of *Exobasidium* is septate and dicaryotic, and that the basidiospores are monocaryotic and haploid. My earlier experiments have shown that *E. vaccinii* "vit.id." is homothallic, with the dicaryotic and pathogenic phase only on the host. To decide whether hybridization and recombination can occur between two *Exobasidium* species, crossing experiments were made, on the host. *E. vaccinii* "vit.id.", which utilized maltose (maltose$^+$) but did not grow on a medium with pyrimidine (thiazole$^-$) as the only vitamin, was mixed with another *Exobasidium* species which was maltose$^-$ and thiazole$^+$. A conidial suspension with conidia of both species was injected into young buds of *Vaccinium vitis-idaea*. After one month there were many disease symptoms, all with the characteristic appearance of *E. vaccinii* "vit.id.". From sporeprints of the diseased leaves on agar, pieces of agar abundant in basidiospores were transferred to agar slants with minimal medium (medium 3 with glucose and thiamine replaced by maltose and pyrimidine). Each leaf was represented by one tube. After three weeks on minimal agar, which could support growth only of maltose$^+$–thiazole$^+$ segregants, if present, large inocula were transferred to test tubes with minimal nutrient solution for turbidimetric measurements of growth. A mixture of the two parent species which had also been cultured on minimal agar for three weeks was used as control. In the growth test they were cultured on minimal medium or on minimal medium + thiamine. As can be seen from Fig. 20 the controls grew both in complete and in minimal medium. The growth in minimal medium of the mixed parent species was effected by syntrophism. Separate colonies of the two species on agar minimal medium also stimulated each other to growth. In contrast, the fungi isolated from the plants inoculated with a mixture of the two species did not grow in minimal medium.

Homothallic fungi produce recombinant progeny by means of sexual and para-

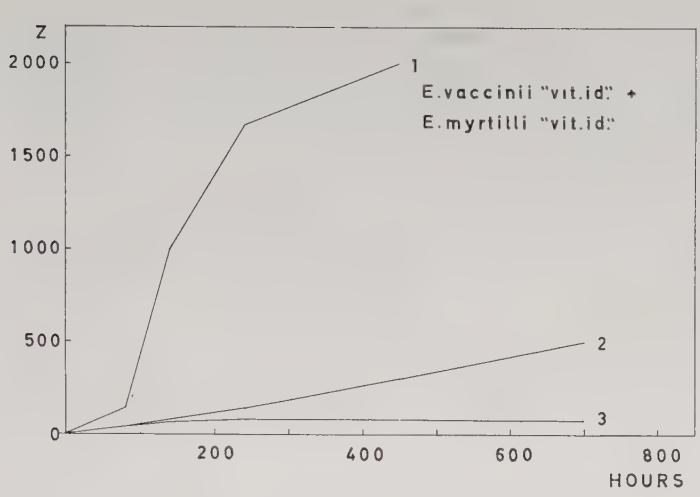


Fig. 20. Test for interspecific recombinations. 1. and 2. represent growth curves of a mixture of conidia from two species, *E. vaccinii* "vit. id." and *E. myrtilli* "vit. id.", cultured in complete and minimal medium, respectively; 3. a growth curve of a population of basidiospores isolated from plants inoculated with a mixture of the two species. This curve represents the collection, out of 13, which gave the best growth.

sexual processes. Some of these recombinants could be expected to grow on minimal medium. However, even if no recombinations had occurred and the progeny consisted exclusively of representatives of the parent types, there might be growth on minimal medium by means of synthrophism. Absence of growth indicates that no hybridization between the two *Exobasidium* species could have occurred. The systemic species used in crossing experiments with *E. vaccinii* "vit. id." had longer incubation time than the gall-producing species. The inoculated plants were observed for 3 months; during this time there were no other disease symptoms than the usual galls of *E. vaccinii* "vit.id.".

The results of the crossing experiments and the number of the tested collections can be seen in Table 11. There was insufficient material for definite conclusions

Table 11. Studies on interspecific hybridization.

Crosses	Number of diseased leaves tested	Observed hybridization
E. vaccinii "vit. id." + *E. cassiopes*	5	None
E. vaccinii "vit. id." + *E. Karstenii*	5	None
E vaccinii "vit. id." + *E. myrtilli* "vit. id."	13	None
E. vaccinii "vit. id." + *E. myrtilli* "myrt."	14	None

to be drawn, but the results indicate that interspecific hybridization did not take place in the studied species, as in no case did growth occur in minimal medium.

The growth test in minimal medium must not be extended for too a long period, because of the adaptation to thiamine synthesis (p. 32) which regularly occurs after 1500–2000 hours.

This technique might be a tool for further studies of reproductive isolation of the different taxa and gives valuable information about the validity of species.

F. Taxonomic aspects

Most of the reported work has concerned problems of method, and there are few published results of cross-infection experiments. The experiments in the present investigation have shown that *E. vaccinii* "vit.id." is strongly specialized to *V. vitis-idaea*, where it causes its typical disease symptoms, and that its conidia were not pathogenic to other related hosts. Further, a changed host, e.g., a hybrid between *V. myrtillus* and *V. vitis-idaea*, does not function as host plant for *E. vaccinii* "vit.id.". This agrees with earlier observations that both the physiology and morphology of this host-race was specialized and stable, and different from other host-races. Graafland's experiments (1960) also showed that in spite of the similar disease symptoms of *E. vaccinii* and *E. japonicum* they could not cross-infect Azalea cultivars and *V. vitis-idaea*, respectively.

The experiments have also shown that there are differences in pathogenicity of the host-races to the susceptible *V. vitis-idaea*. *E. vaccinii* "vit.id." was the only host-race that caused a high percentage of infections in field experiments.

As the virulence of other host-races than *E. vaccinii* "vit.id." to their host-plant is low, the results so far obtained are indefinite. The experimental conditions must be improved before the degree of specialization of the fungus can be determined by cross infection experiments.

VII. SEROLOGICAL STUDIES

A. Introduction

Exobasidium strains causing similar disease symptoms and parasitizing the same host species have in the present investigation been called a host-race. A large number of strains belonging to different host-races has been studied, and in the preceding sections it was stated that the members of one host-race had a characteristic morphology and physiology. My inoculation experiments and Graafland's (1960) indicated that the host-races were specialized and it seemed that the sexual phase occurred only on the host. This character, together with homothallism, preserves the characters of the host-race constant and unmixed with other host-races. The methods used are valuable for the identification of taxa in *Exobasidium*, but less useful for giving information about the relationship between the taxa or about the taxonomic position of the genus.

To get a better insight into these problems, serological analyses have been carried out. By immunodiffusion it is possible to identify the occurrence of antigen-antibody reactions and to differentiate closely related proteins or polysacharides of a fungus, on the basis of their immunological specificity. Since the development of the double diffusion plate test (Ouchterlony 1948), this technique has been an important taxonomic tool. Most studies, however, have been on bacteria, but as can be seen from references and surveys of literature in Ouchterlony 1958, Tempel 1959, Crowle 1961, Biguet *et al.* 1962, and Dyson and Landay 1963 many fungi have also been successfully studied.

Savile (1959) summarized the taxonomic position of the *Exobasidiaceae* as follows: "The family may be placed in the Heterobasidiomycetidae according to one's definition of the limits of these two groups. I have previously (5) treated it as the most primitive of the Homobasidiomycetidae and nearly on the line of origin of this group from the Heterobasidiomycetidae. Talbot (9) would include it in the latter group, which he defines to include holobasidiomycetes in which the basidiospores germinate by repetition."

In the present serological investigation not only the relation between the host-races of *Exobasidium* will be discussed, but also the relationship between this genus and some members of the Heterobasidiomycetidae and the Homobasidiomycetidae. In selection of species of other genera, no particular phylogenetic principles have been followed, and pure cultures available in the Institute's collection were used. The *Ustilago* and *Tilletia* species, which were of interest as the basidiospore germination and subsequent growth is similar to that of some *Exobasidium* species, were isolated from diseased plants.

B. Material and methods

1. PRODUCTION OF ANTIGEN AND ANTISERUM

The species of *Exobasidium* listed in Table 1 are represented in this investigation by one or more strains from each host-race. In addition, pure cultures of the following species were also tested:

Daedalea unicolor Bull. ex Fr.	Clavaria mucida Pers. ex Fr.
Fomes variegatus (Secr.) Sacc.	Tilletia caries Tul.
Corticium laeve Pers.	Ustilago avenae (Pers.) Rostr.
Peniophora fraxinea (Pers.) Lll	Calocera viscosa (Pers. ex Fr.) Fr.

All fungi were cultivated on medium 3, pH 4.2, with vitamins added. As some of the fungi of other genera than *Exobasidium* grew poorly on this medium, these were also grown on medium 4, pH 5.0, (p. 14). *Tilletia caries*, which did not grow on either of these two media, was cultivated on MT 1 medium (Zscheile 1951). Cultivation was at 20°C in one litre Erlenmeyer flasks with continuous shaking. The fungi were harvested in the linear growth phase, and their conidia and mycelia were collected by centrifuging and washed twice with sterile distilled water. The washed material was then frozen at −20°C, freeze-dried, and then ground in a mortar together with NaCl. When the cells had become disintegrated, water was added. The weight-proportions between water, salt and dried fungus were 100:0.9:5 for the production of antigens for immunization, and 100:0.9:2 for the diffusion test.

The suspension of the ground fungus used in the tests was left at 5°C overnight, and then centrifuged to give the clear antigen extract, which was stored at −20°C.

Specific antisera were obtained from rabbits after injections of disintegrated conidia or mycelia suspended in a saline solution. One ml, corresponding to 50 mg dry weight of the fungus, was injected intramuscularly once a week and the animals were bled regularly. The blood clot was removed from the sample by centrifugation and the sera were deep frozen until used. Sera obtained 3–5 months after the first injection were used.

2. IMMUNODIFFUSION TECHNIQUE

The double diffusion plate technique (Ouchterlony 1948) was used, but the tests were carried out on microscope slides. The gel was prepared by dissolving 1.1 % ion agar (Oxoid no. 2) in veronal buffer at pH 7.4 (p. 14). The agar was cooled to 70°C and merthiolate was added to a final concentration of 1:10.000. 2.2 ml of this agar was spread evenly over the slide. The gels were kept in a humid atmosphere and stored at 5°C overnight until used, so that the agar solidified. Before the tests, wells are cut in the agar. The double diffusion test was used to

determine the optimal concentrations of antigen and sera to give a maximum number of precipitation lines.

Usually it was necessary to concentrate the antigen 4–10 times in "Kollodiumhülsen zur Liquoranreicherung" (Membranfiltergesellschaft Göttingen) and to dilute the sera, to obtain optimal concentrations.

In the study of the serological relationships, both antigen-antibody reactions of different strains belonging to the same host-race, and reactions between different host-races, or between *Exobasidium* and other genera, were compared. The tests were carried out at 20°C. The plates were studied daily and the precipitation bands were photographed in dark-field illumination on Adox KB 14 film developed in Kodak D 76.

C. Results

The results of the tested serological relationships are summarized in Table 12. In this test most *Exobasidium* host-races were represented, together with some other species. The left part of the table comprises the host-races that were compared with both homologous and heterologous sera, and each value is based on at least four replicates in which the antigen/antisera proportions were varied. In the right part of the table the results are based on at least two replicates and the comparisons were made with heterologous sera. The figures in the table denote the number of precipitation lines that occur between the antigens of a host-race and an antiserum. These figures may not be interpreted as an absolute measure of the serological relationship, as sometimes a line was very weak, probably because of too low a concentration. In some of these cases, a line could be seen only in one out of four tests; in others they may have escaped observation. Besides the homologous antigen, a heterologous antigen may sometimes react with the same antibodies and form a precipitation line, and this causes partial intersection (Ouchterlony 1958). In the present tests partial intersections have also occurred. In the table no distinction between the two reactions (identical and partly identical) has been made, since it may be supposed that the heterologous antigen is very closely related to the homologous.

In the report of the serological results only the most obvious will be further illustrated.

The reactions of antigens of different strains of the same host-race towards an antiserum, produced against one of the strains, were compared with one another. Two to four precipitation lines were formed, and the strains belonging to the same host-race had identical lines (Plate 6 a and b). The number of precipitation lines sometimes varied, when antisera from different rabbits were compared to the same homologous antigen mixture. In *E. myrtilli* "vit.id." and *E. vexans* (Table 12) this variation was caused by different relations between the concentration of the antibodies. Plate 6 c shows that the reduced number

Table 12. The number of precipitation lines arising in double diffusion tests between various antigens and antisera.

| Antigen | | |

of lines in one of the antisera towards *E. myrtilli* "vit.id." depends on the fact that more lines were precipitated on the same place, and therefore the separate lines cannot be seen.

As can be seen in Table 12 the host-races of *Exobasidium*, with the exception of *E. vexans*, constitute a serologically related group, but they are not closely related to the other tested genera of Hetero- or Homobasidiomycetidae. *E. vexans*, on the other hand, shows close agreement with the *Tilletiopsis* strains (Plate 6 d), while their antisera only reacted with *E. myrtilli* "myrt." of the *Exobasidium* host-races (Table 12 and Plate 6 e). Of the studied *Exobasidium* host-races, *E. angustisporum* gave the faintest precipitation lines. *E. cassiopes* and *E. japonicum* are serologically related, with at least 2 antigens in common (Plate 6 f), but *E. japonicum* also shows a close relationship to *E. myrtilli* "vit.id." (Plate 6 c). The other host-races of *E. myrtilli* are serologically closely related but their antigenic composition diverges distinctly from that of *E. myrtilli* "vit.id." (Plate 7 a). *E. myrtilli* "Andr." and *E. Karstenii*, which both cause systemic attacks on *Andromeda polifolia*, have different antigens, while *E. Karstenii* has a somewhat closer relationship to *E. vaccinii* "vit.id." (Plate 7 b).

In spite of the great similarities in disease symptoms, the host-races of *E. vaccinii* are antigenically heterogenous (Plate 7 c). On the other hand, host-races with dissimilarities in disease symptoms, morphology and physiology can be serologically closely related, e.g., the two host-races on *Arctostaphylos uva-ursi*, *E. vaccinii* "Arct." and *E. uvae ursi* (Plate 7 d).

In contrast to the parent strain, the mutant of *E. myrtilli* "vit.id." utilized maltose, lacked pigment production and had a rapid growth. Plate 7 e shows that there is only a slight serological relationship between this mutant and the parent strain.

The test has also shown that *E. vaccinii* 6–8, reported by Sundström (1960) were contaminating fungi with no antigenic relationship to *Exobasidium*.

D. Taxonomic aspects

The serological results agree well with the information obtained from physiological and morphological studies, with regard to the great similarity of strains within the same host-race and the differences between the host-races. In most cases there is a change in morphological and physiological, as well as in serological characters from host-race to host-race. Only a few exceptions to this pattern have been found: *E. myrtilli* "Andr.", "myrt." and "ulig." have an almost identical antigenic composition but differences in physiological, morphological and cultural characters; in contrast, *E. Karstenii* and *E. myrtilli* "myrt." are physiologically similar but have different serological characteristics.

Thus, in most cases the differentiation of host-races has resulted in physio-

logical and morphological, as well as serological divergence. The relationships between the established characteristics of the host-races and the host-parasite relations are, however, not easily intelligible. Nevertheless, the separation of different host-races is constant and clear-cut and deserves taxonomic attention. It is probably premature to decide upon the taxonomic rank of the host-races now. First, more inter-host inoculations should be carried out and crosses between genetically marked host-races on the plant should be made, if possible.

An indication of the genetic background to the differences between host-races may be obtained from the mutant of *E. myrtilli* "vit.id.". In contrast to the "wild type", the mutant is able to utilize maltose, lacks pigments and grows rapidly. The serological behaviour is also strongly changed by the mutation; the reaction of antigens from the mutant against antisera of the wild type is not stronger than the reaction of antigens of other host-races of *E. myrtilli*. Although it cannot be established with certainty that the mutant is the result of a one point mutation, it is apparent that physiological and serological differences comparable to those found between different host-races can arise as a result of a simple genetic change.

Observations reported by Gross (1958) and by Gross and Fein (1960) show that large changes may occur through a single mutation. Accumulation of a metabolite at a block in a metabolic pathway in *Neurospora* caused induction of detectable levels of enzymes which were not found in the wild type. In a similar way small genetic changes may induce new pathways, which result in large changes in physiological and antigenic characters in *Exobasidium*. Similarly complex effects may be expected if the blocked enzyme takes part in more reactions.

The similarities between the strains of *E. vexans* and the other host-races of *Exobasidium* are slight in all respects. The morphological, cultural and serological similarities to species of the genus *Tilletiopsis* suggest a close relationship between *E. vexans* and *Tilletiopsis*. As long as the identity of the material of *E. vexans* with the fungus described by Massee (1898) is not definitely established, no conclusions as to the taxonomic position of the species should be drawn. However, the agreement between observations of production of "basidiospores" outside the host, which is atypical of the genus *Exobasidium*, both in my cultures of *E. vexans* and in Japanese material (Ezuka 1955), suggests that the identification of my strains is correct. On the other hand, antigenic similarities between the genus *Tilletiopsis* and the other species of *Exobasidium* indicate that there must be at least some slight relationship between the genera. Otherwise, the serological studies do not indicate any relation between *Exobasidium* and other genera of Homo- or Heterobasidiomycetidae studied. The precipitation lines in *Tilletia* and in *Corticium* were very faint and diffuse.

E. E. myrtilli "Andr.", a new host-race

This new host-race causes systemic attacks on *Andromeda polifolia* like *E. Karstenii* Sacc. and Trott. It is distinguished from *E. Karstenii* as it causes less broadening of the leaves and can be seen in the small shoots, usually arising from the stem and not so often from the rhizome. The attacked leaves are light red with green dots of uncoloured tissue, forming a mosaic pattern. This form also differs from *E. Karstenii* in physiological and morphological characters. The basidiospores of *E. Karstenii* ($2.5-3\times14-17\ \mu$) produce secondary spores on germination, while the basidiospores of *E. myrtilli* "Andr." ($2\times10-12\ \mu$) form mycelia.

The mode of germination and the size of the basidiospores, as well as the antigenic similarities, indicate a close relationship with some of the host-races of *E. myrtilli*, and justify the inclusion of this new form on *Andromeda polifolia* in *E. myrtilli*.

F. Some general conclusions

Since the morphological, physiological, and serological characters and the relationship of the host-races have been discussed in the chapters dealing with taxonomic aspects, only some aspects of the variability of taxonomic and pathogenic characteristics will be taken up here.

The results of the present investigation on *E. vaccinii* "vit.id." and Graafland's on *E. japonicum* (1960) showed that the conidia and the basidiospores are uninucleate, that dicaryotization occurred only on the host, and that the species are homothallic. These conditions influence the nature of the parasitism and of the variation occurring within species and host-race.

In isolates from fungi which have multinucleate and heterocaryotic conidia and basidiospores, changes in the ratio between the numbers of each type of nucleus or segregations occur and cultures with varying characters result (Hansen 1938). This source of variation is excluded in *Exobasidium* because of the uninucleate cells. Other sources of variation are mutations and enzymatic inductions. In *Exobasidium*, mutations are rarely observed and most of them are probably not of any survival value in the balanced host-parasite system. Variation by enzymatic induction can be considerable within the same isolate if the organism is studied under different conditions and with varying incubation time. In a previous study of *Exobasidium* (Sundström 1960), *E. myrtilli* "myrt." and *E. Karstenii* were considered thiamine heterotrophic. Later studies have shown that in all strains of *E. myrtilli* "myrt." and in some strains of *E. Karstenii*, thiamine synthetic activities are induced when the incubation time is extended. With an extended incubation time the number of different carbohydrates utilized also increased.

In vitro sexual and parasexual recombination seem to be excluded, at least in

the conidial forms of *Exobasidium*, as all evidence indicates that conidia in culture are haploid and uninucleate.

Whether there are recombinations *in vivo* in *Exobasidium* is not known, but investigations of another homothallic pathogen have shown that recombinant biotypes occur (Björling 1951).

The homothallic *Exobasidium* spp. maintain the genotype constant from one season to the next. According to Griffith (1958) this property and the ability of the host to inbreed and propagate clonally is important for the maximum degree of physiological specialization. Both these conditions occur in the host-parasite system of *Exobasidium*.

Within the genus *Exobasidium*, there are a large number of separate biological races each with a characteristic physiology and morphology, and often also characteristic antigenic properties. The exact similarity in these characters between the isolates of the same host-race from different geographic areas, shows that hybridization between the host-races is lacking or is at least unusual.

SUMMARY

In the present investigation the strains of *Exobasidium* parasitizing the same host-species and causing similar disease symptoms are called a host-race. Pure cultures of 72 *Exobasidium* strains, belonging to nine species representing 16 host-races, have been studied. One new host-race on *Andromeda polifolia* has been described. This paper reports (I) physiological studies carried out to establish optimal cultural conditions, (II) a comparative taxonomic study of physiological (1), morphological (2), pathogenic (3), and serological (4) characters.

I. In the physiological studies, growth in synthetic liquid media was determined by turbidity or dry weight measurements. For most species the pH range 3.0 to 6.0 is optimal for growth, whereas growth is poor in alkaline media. The temperature tolerance of the host-races is specific, with maximum temperatures ranging from 23 to 29°C (*E. vexans* 34°C). The optimum temperatures vary from 19 to 25°C. All strains utilize ammonium tartrate, asparagine and potassium nitrate as nitrogen sources. Glucose and sucrose were the best carbon sources for growth. Some host-races are autotrophic for all vitamins, while others require either thiamine or its pyrimidine moiety. In *E. vaccinii* an adaptation to thiamine synthesis occurs; this is caused by mutation or by enzymatic induction. In the latter case, the growth starts regularly after 1000 hours (in *E. vaccinii* "myrt.") in the thiamineless pyrimidine-glucose medium at pH 4.2, and after 1500–2000 hours (in *E. vaccinii* "vit.id."). This enzyme induction is accelerated by lowering the pH to 3.0 and by replacing glucose with oxalacetic acid. The induction also occurs in media without thiamine or with thiazole only, but in these media it was not favoured by low pH or by oxalacetic acid.

II. 1. Physiological activities that can be used as taxonomic characteristics of *Exobasidium* are the synthesis of thiamine, and the utilization of xylose, cellobiose, maltose, lactic acid, citric acid, arbutin, salicin, dulcitol, and hydroquinone (see Table 8 and Fig. 16 p. 42–43). Chromatographic analyses and growth studies have shown that growth in arbutin media is determined by the capacity of the strains to split arbutin, their tolerance towards and their power to assimilate the toxic hydroquinone. Hydroquinone is utilized by two host-races.

2. The size of the basidiospores, the mode of germination and the morphology of the colonies on agar was studied. With only a few exceptions each host-race shows constant and characteristic morphological characters. Outside the host, some host-races of *Exobasidium* grow in a mycelial state and others in a conidial

state. In the host, conidial growth is regularly transformed into mycelial growth. The conidial growth outside the host is hardly affected by nutritional or environmental conditions nor by extracts from the host plant. The host-races with mycelial growth are less stable; sulphydryl compounds or appropriate chelators can transform them into a conidial state.

3. *E. vaccinii* "vit.id." is homothallic, since infections leading to hymenium formation are caused on *Vaccinium vitis-idaea* by a uninucleate monospore culture. Inoculations with *E. vaccinii* "vit.id." on hosts of the other host-races, or with the other host-races on *V. vitis-idaea*, gave no attacks. Inoculations were made on mature plants, sterile seedlings and tissue cultures of the host-plant. Infection of mature plants was most successful, while the tissue cultures were not infected.

4. The serological studies show that strains of the same host-race have identical antigens, while there are large antigenic differences between most host-races.

E. vexans is only distantly related to the other host-races of *Exobasidium*. On the other hand, there is a close antigenic relationship to *Tilletiopsis*, with which it agrees in morphology, but differs in physiology. The question whether the fungus called *E. vexans* in the present investigations is the fungus causing blister blight in tea, or whether it has been contaminated with the *Tilletiopsis* species at the time of isolation, is not settled.

A physiological mutant of *E. myrtilli* "myrt." was studied. The mutant which could utilize maltose also had more vigorous growth and lacked pigment-synthesizing capacity. Through this mutation, the antigens of the mutant were altered, so that the mutant showed only distant serological relationship to the parent strain.

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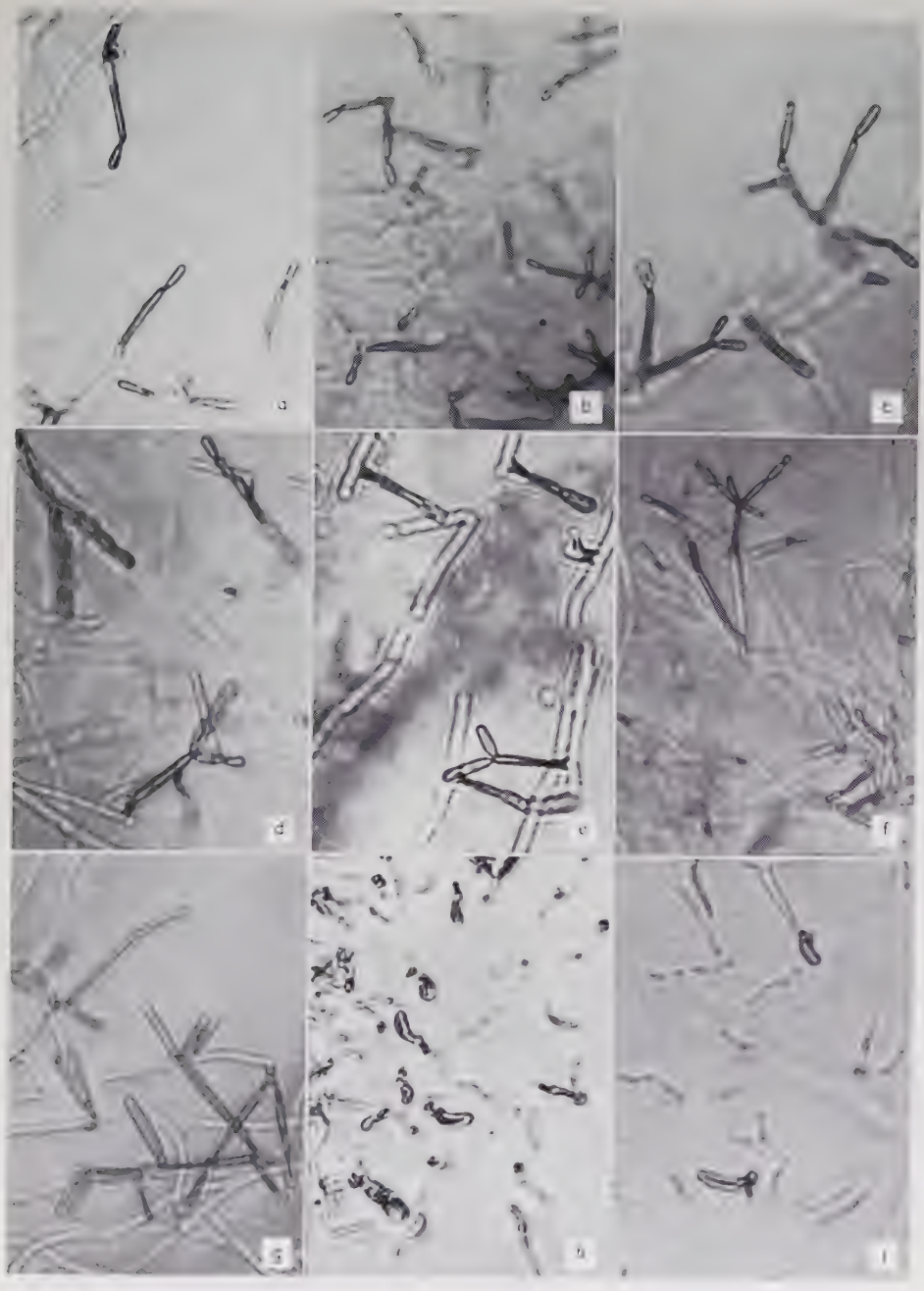


Plate 1.
Conidia formed from mycelia of *E. myrtilli* "Andr." (a), *E. myrtilli* "myrt." (b), *E. myrtilli* "ulig." (c), *E. myrtilli* "vit. id." (d), *E. japonicum* (e), Mutant of *E. myrtilli* "vit. id." (f), *E. cassiopes* (g), *Tilletiopsis* sp. (h), *E. vexans* (i); (× 600).

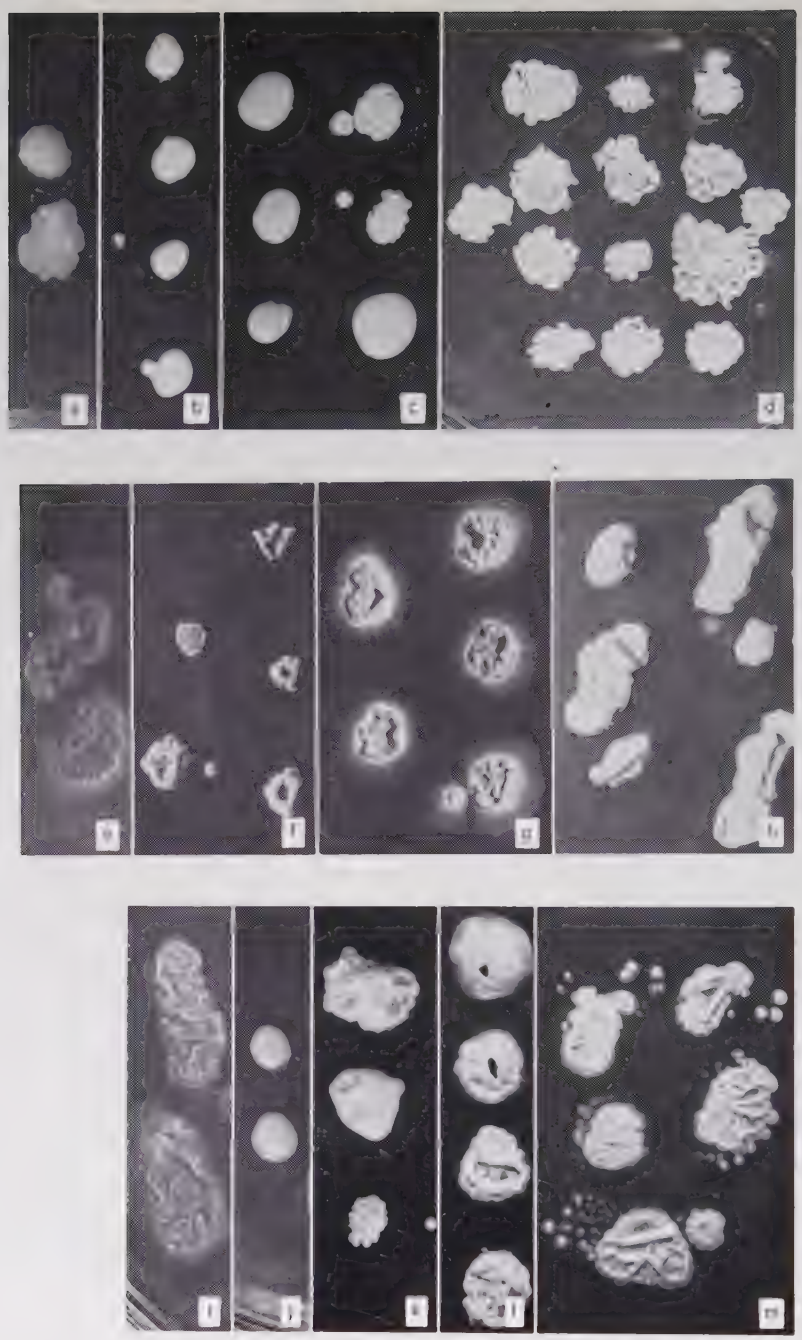


Plate 2.

The appearance of colonies cultivated 20–30 days on agar medium (Medium 3; pH = 5.0). Each host-race is represented by different strains. *E. vaccini* "Arct." (a), *E. vaccinii* "oxyc." (b), *E. vaccinii* "ulig." (c), *E. vaccinii* "vit. id." (d), *E. myrtilli* "Andr." (e), *E. myrtilli* "myrt." (f), *E. myrtilli* "vit. id." (g), *E. Karstenii* (h), *E. cassiopes* (i), *E. uvae ursi* (j), *E. angustisporum* (k), *E. japonicum* (l), *E. vexans* (m).

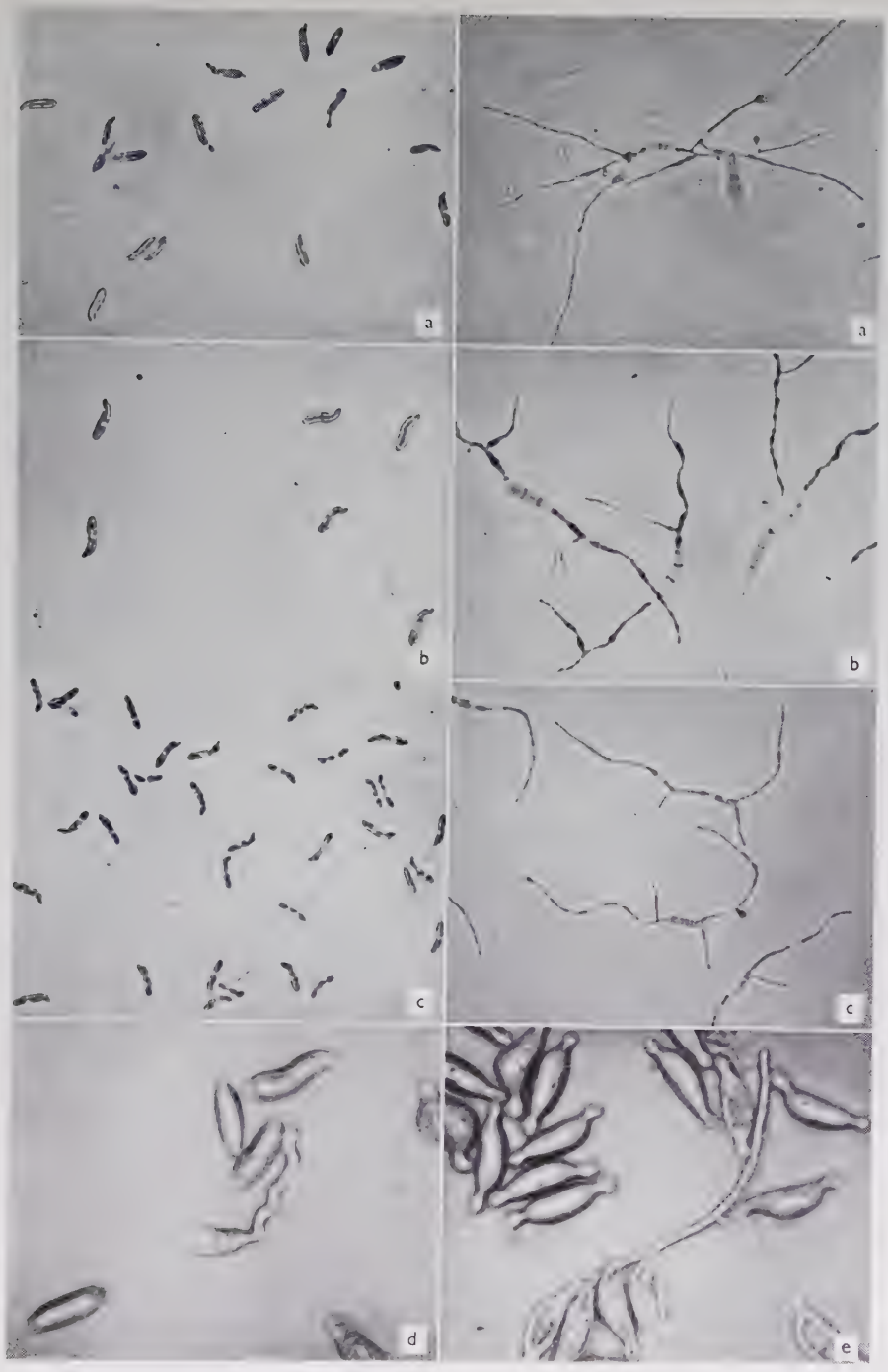


Plate 3.

Basidiospores of *E. myrtilli* "Andr." (a), *E. myrtilli* "myrt." (b), *E. myrtilli* "vit. id." (c), *E. vaccinii-uliginosi* from *V. vitis-idaea* (d), *E. vaccinii-uliginosi* from *V. uliginosum* (e). The left photos of *E. myrtilli* were taken after 3 hours, the right ones after 30-40 hours. The basidiospores of *E. vaccinii-uliginosi* were photographed after 4 months; (× 425).

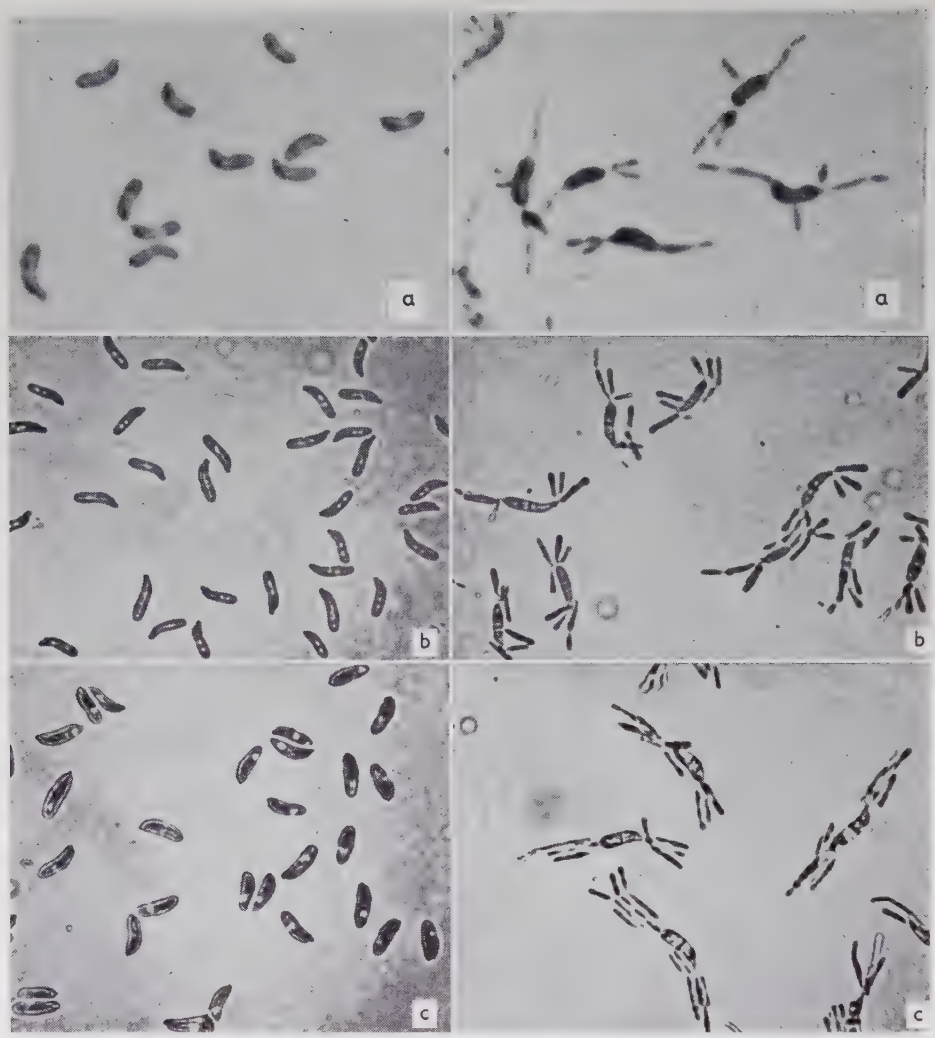
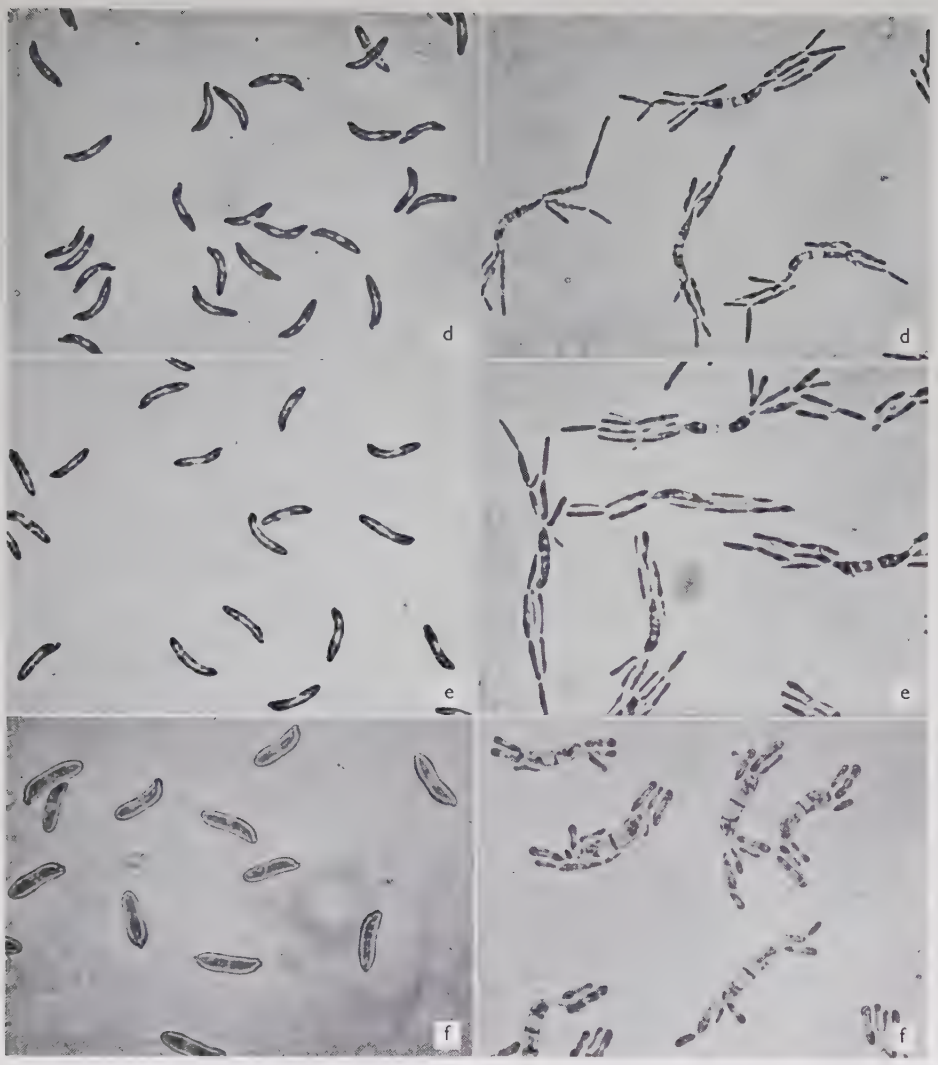


Plate 4.
Basidiospores of *E. vaccinii* "Arct." (a), *E. vaccinii* "oxyc." (b), *E. vaccinii* "ulig." (c), *E. vaccinii* "vit. id." (d), *E. Karstenii* (e), *E. uvae ursi* (f). The left photos were taken after 3 hours, the right ones after about 20–30 hours; (× 425).


d
d
e
e
f
f

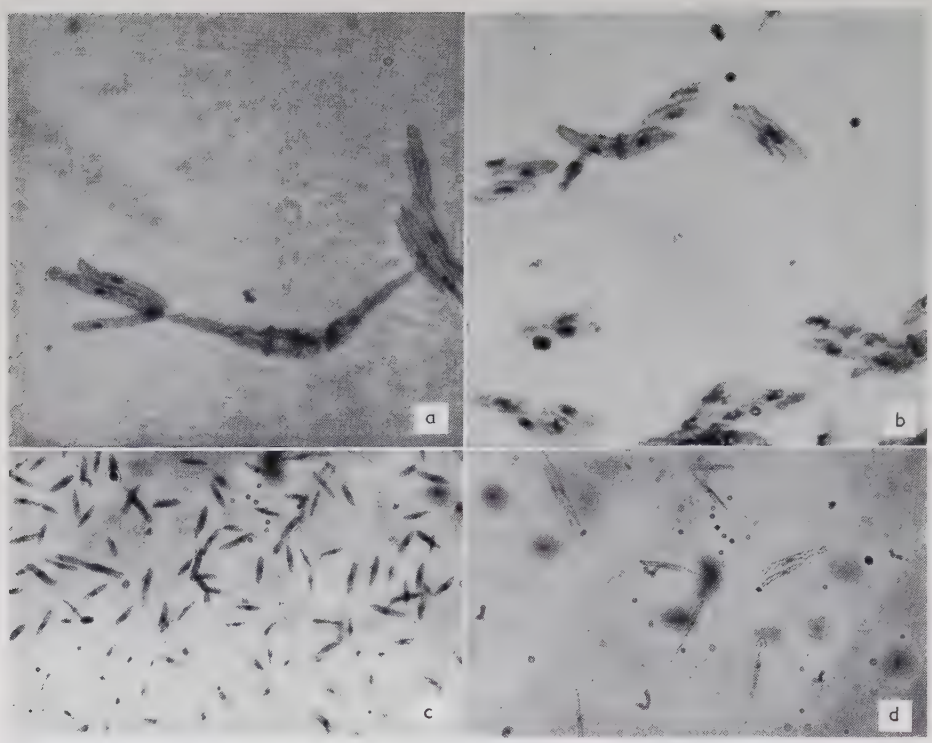


Plate 5.
Uninucleate conidia of *E. Karstenii* (a), *E. vaccinii* "vit. id." (b), *E. vaccinii* "Arct." (c), and *E. cassiopes* (d), stained with Azure A. In photos a and b where germinating basidiospores were stained, the conidia and, respectively, one and two deformed and septated basidiospores can be seen. In photos c and d a conidial suspension was stained.

Plate 6.

1. a. Double diffusion test comparing antigens of three strains of *E. Karstenii* against an antiserum of *E. Karstenii* 1.
   b. Double diffusion test comparing antigens of four strains of *E. myrtilli* "vit. id." and two strains of *E. myrtilli* "myrt." against an antiserum of *E. myrtilli* "vit. id." 3.
   c. Double diffusion test comparing antisera of *E. cassiopes*, *E. japonicum* and two different antisera of *E. myrtilli* "vit. id." against antigens of *E. myrtilli* "vit. id.".
   d. Double diffusion test comparing antigens of *E. vexans* and three strains of *Tilletiopsis* against an antiserum of *E. vexans*.
   e. Double diffusion test comparing antigens of *Tilletiopsis*, *E. vexans*, *E. myrtilli* "myrt.", *Tilletia caries* and *Corticium laeve* against an antiserum of *E. vexans*.
   f. Double diffusion test comparing antigens of *E. japonicum* and *E. cassiopes* against an antiserum of *E. japonicum*.

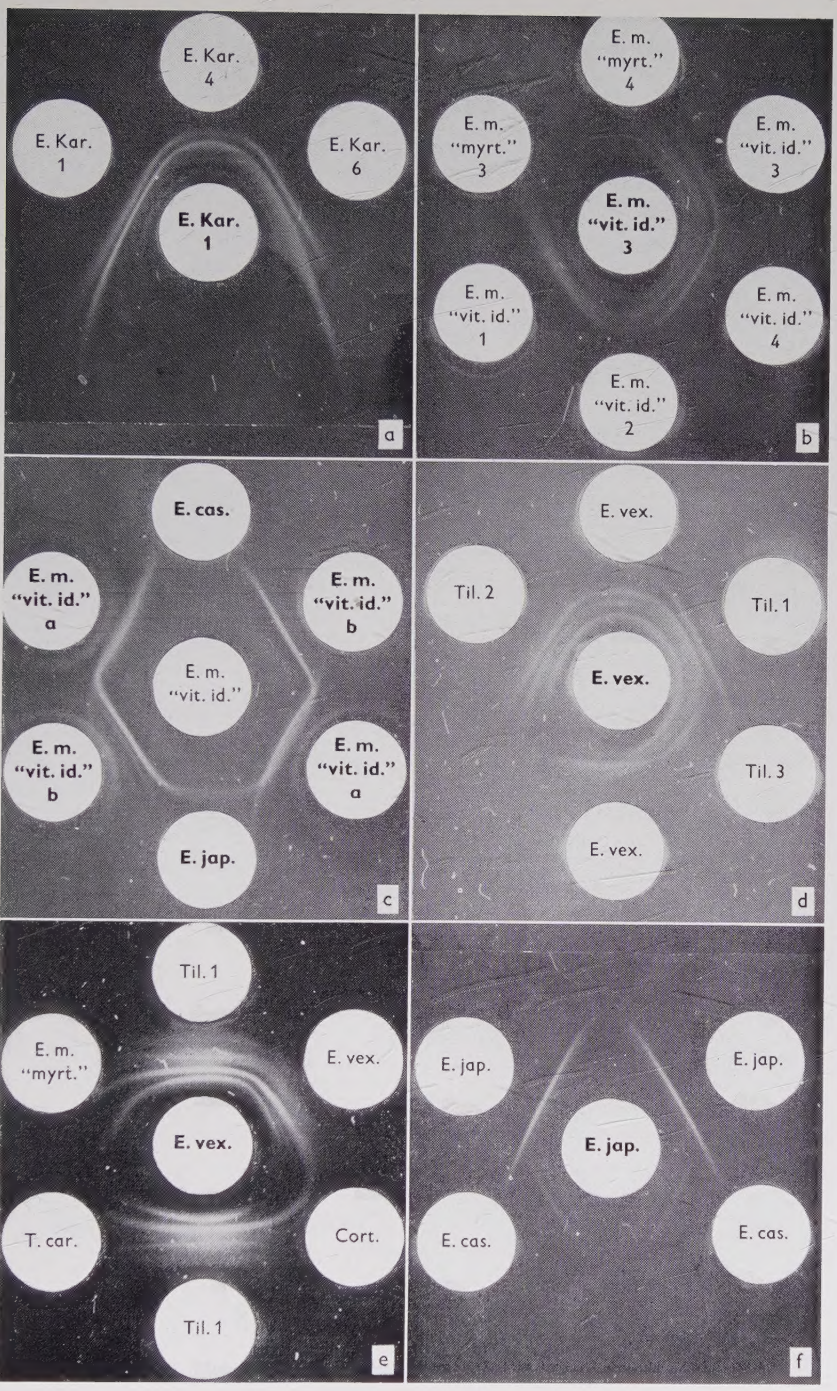

E. Kar. 4
E. Kar. 1
E. Kar. 6
E. Kar. 1
a
E. m. "myrt." 4
E. m. "myrt." 3
E. m. "vit. id." 3
E. m. "vit. id." 3
E. m. "vit. id." 1
E. m. "vit. id." 4
E. m. "vit. id." 2
b
E. cas.
E. m. "vit. id." a
E. m. "vit. id." b
E. m. "vit. id."
E. m. "vit. id." b
E. m. "vit. id." a
E. jap.
c
E. vex.
Til. 2
Til. 1
E. vex.
Til. 3
E. vex.
d
Til. 1
E. m. "myrt."
E. vex.
E. vex.
T. car.
Cort.
Til. 1
e
E. jap.
E. jap.
E. jap.
E. cas.
E. cas.
f

Plate 6.

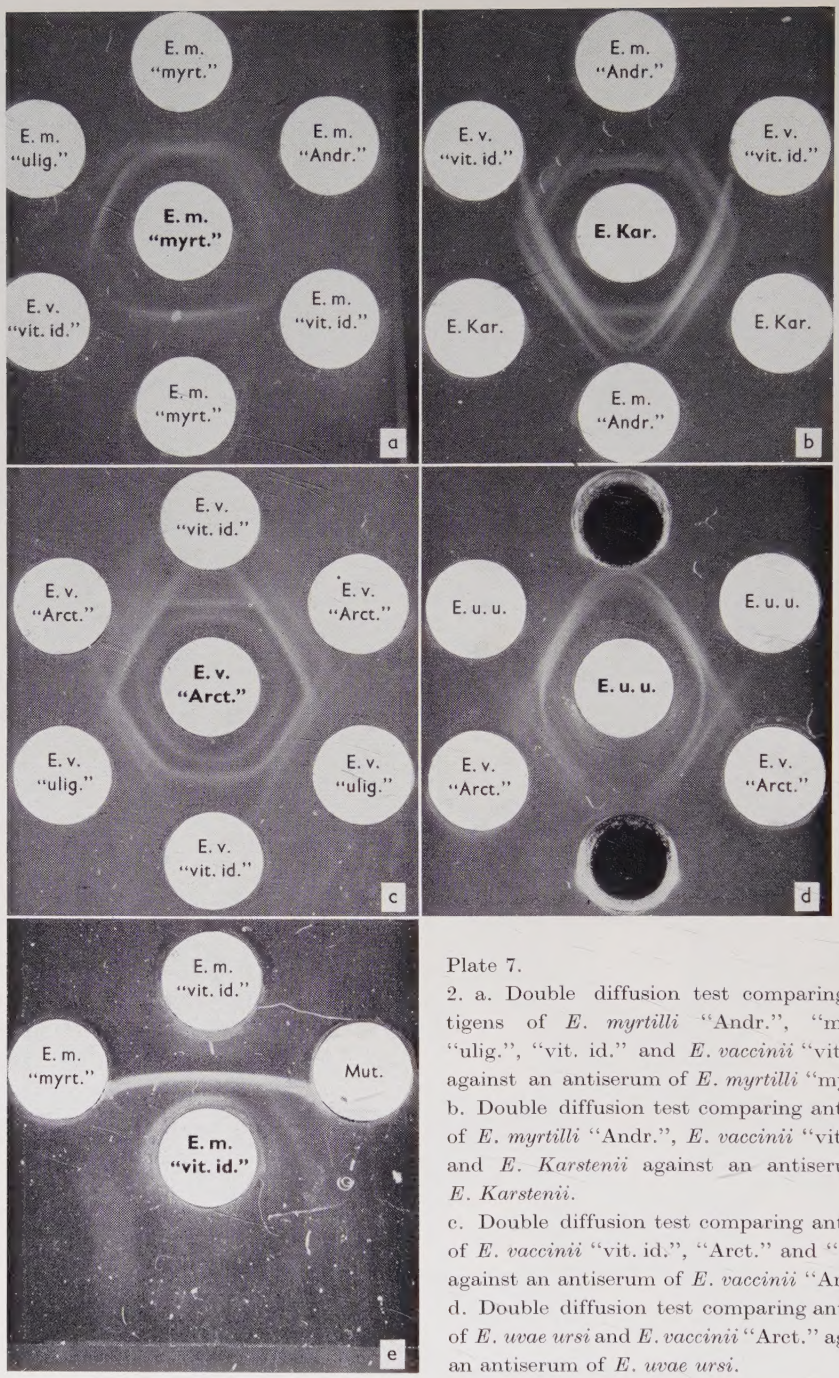


Plate 7.

2. a. Double diffusion test comparing antigens of *E. myrtilli* "Andr.", "myrt", "ulig.", "vit. id." and *E. vaccinii* "vit. id." against an antiserum of *E. myrtilli* "myrt.".

b. Double diffusion test comparing antigens of *E. myrtilli* "Andr.", *E. vaccinii* "vit. id." and *E. Karstenii* against an antiserum of *E. Karstenii*.

c. Double diffusion test comparing antigens of *E. vaccinii* "vit. id.", "Arct." and "ulig." against an antiserum of *E. vaccinii* "Arct."

d. Double diffusion test comparing antigens of *E. uvae ursi* and *E. vaccinii* "Arct." against an antiserum of *E. uvae ursi*.

e. Double diffusion test comparing antigens of *E. myrtilli* "vit. id.", a mutant of *E. myrtilli* "vit. id.", and *E. myrtilli* "myrt." against an antiserum of *E. myrtilli* "vit. id.".

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